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by

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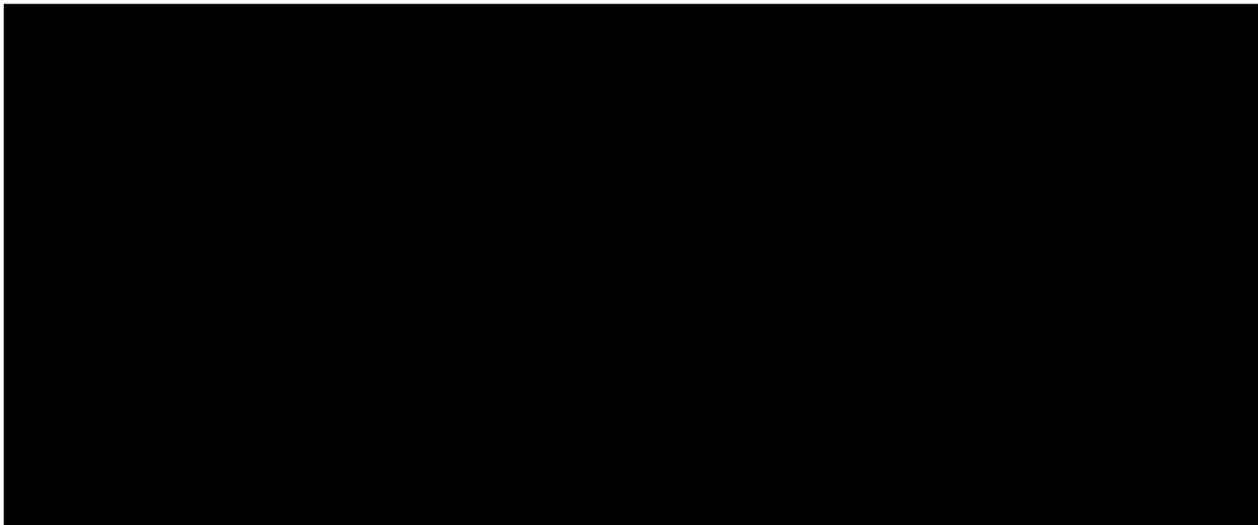
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Participation of early response genes in the response of B cells to antigen receptor signaling

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ABSTRACT

Cross-linking of the antigen receptor on B lymphocytes induces intracellular signaling events that regulate B cell growth and differentiation. Antigen receptor stimulation also induces the expression of early response genes, which are believed to be mediators that help translate biochemical signaling reactions into long-term cellular changes. In the first chapter, I report that expression of four of seven early response genes first identified in fibroblasts was induced by antigen receptor cross-linking of resting splenic B cells. Two of these genes encode transcriptional regulators and one encodes a protein phosphatase. In two B lymphoma cell lines examined, either two or three of these four genes were induced. Thus, a subset of the early response genes identified in serum-stimulated fibroblasts was also induced by antigen receptor stimulation in B lymphocytes, and this subset was further subdivided among transformed types of B cells. Almost all of the inductions appeared to be mediated by the phosphoinositide signaling pathway and in particular by protein kinase C.

In the second chapter, I explore the role of an antigen receptor-inducible early response gene, c-*myc*, in the apoptotic response to BCR signaling in the WEHI-231 cell line. This B lymphoma-derived cell line is a model for the clonal deletion of immature B cells expressing self-reactive antigen receptors.

To test whether the antigen receptor-mediated induction of c-*myc* could itself activate apoptosis of WEHI-231 cells, a hormone-inducible chimeric protein consisting of c-Myc fused to the estrogen-binding domain of the estrogen receptor (MycER) was introduced into these cells. Activation of the MycER protein was found to trigger apoptosis by itself, even under conditions that otherwise permitted proliferation of the cells. In addition, the MycER chimera dramatically accelerated the rate of apoptosis induced by the antigen receptor. These results are consistent with the hypothesis that c-*myc* participates in antigen receptor-mediated apoptosis.

Several observations indicated, however, that antigen receptor signaling does not induce apoptosis simply by causing an elevation of c-*myc* expression. For example, treatment with bacterial lipopolysaccharide, which delays antigen receptor-mediated apoptosis in WEHI-231 cells, did not delay MycER-mediated cell death. In contrast, stable overexpression of the anti-apoptotic proto-oncogene *bcl-2* delayed MycER-mediated apoptosis but not antigen receptor-mediated apoptosis. These observations imply that there is a c-*myc*-independent event that is required for antigen receptor-induced apoptosis. Although it is likely that c-*myc* participates in antigen receptor-mediated apoptosis, its importance for this process remains to be demonstrated. Clearly, a further understanding of the biological roles of c-*myc* and the other antigen receptor-induced early response genes in mediating the responses of B lymphocytes to antigen is an important future goal. In summary, the studies reported here have identified a number of antigen receptor-induced early response genes, have characterized the mechanism of their induction, and have explored the biological importance of one early response gene, c-*myc*, in antigen receptor-induced apoptosis.

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INTRODUCTION

B lymphocytes make the antibodies that perform the humoral function of the immune system. A prerequisite for a functional immune system is that it recognize all unforeseen foreign antigens while avoiding recognition of self antigens. The clonal selection theory of Burnet, the principles of which apply to T cells as well as to B cells, hypothesized that, in order to enable control of the abundance of particular antibodies, each B lymphocyte must make a single unique antibody (1). To detect the presence of specific antigen, the B cell expresses on its cell surface a membrane form of its particular antibody, called membrane immunoglobulin (mIg), which functions as its antigen receptor (2). Antigen binding by mIg initiates processes that ultimately enable B cell clones whose antibodies recognize foreign antigens to reproduce and differentiate into large-scale antibody producers. Conversely, antigen binding by B cells that recognize self antigen leads to the inactivation or elimination of those potentially destructive clones. The details of the complex interactions between antigens, lymphocyte subsets, and other types of cells needed to develop and implement self-tolerant immunity are beginning to be understood (3). Also under intense investigation are the intracellular mechanisms by which lymphocytes carry out antigen-directed processes such as entry into the cell cycle, differentiation, and self deletion.

The B cell antigen receptor functions by two mechanisms

The first role of the B cell antigen receptor is to capture antigen for internalization and proteolysis, generating peptide byproducts that are loaded into the antigen-binding pockets of class II major histocompatibility (MHC) molecules. Peptide-loaded MHC molecules move to the cell surface where

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they serve as the structures recognized by the T cell antigen receptor. By using its mIg to collect antigen, the B cell serves as a highly specific antigen-presenting cell for antigen-specific helper T cells. In its other role, the antigen receptor signals directly to the B cell that it has bound antigen. In this capacity, the antigen receptor serves many functions in the development of the B cell repertoire and in immune responses. Several of these functions are briefly described below.

Immature B cells derive a negative signal from antigen receptor engagement

A key element in the ability of the B cell to distinguish self antigens from foreign antigens is the stage of maturation at which the B cell first encounters antigen (4). If, upon successful rearrangement of its heavy and light chain loci to form a functional antigen receptor, the immature B cell encounters its specific antigen, it "presumes" that that antigen is of host origin. In this event, rearrangement of the light chain genes continues, with the goal of replacing the light chain and perhaps generating a nonreactive antigen receptor. If this process of receptor editing fails to eliminate antigen binding, the continuing mIg signal prevents maturation (5) and can lead to death of the cell (6, 7). Activation-induced cell death of immature B cells is believed to occur by apoptosis (8), which is a suicide mechanism by which the cell dismantles itself while maintaining plasma membrane integrity (9). The post-cellular apoptotic structure quickly undergoes phagocytosis by macrophages and is thereby removed from the circulation, thus avoiding the release of its inflammation-inducing intracellular contents.

Mature B cells can respond positively to antigen but require a second signal

The immature B cell that survives the period of elevated sensitivity to mIg-induced self deletion differentiates into a mature B cell, which is now capable of responding positively to a signal from mIg. A generally accepted theory states that two signals are needed for activation of a mature B cell: the first comes from the antigen receptor and the second comes primarily, in the case of a T cell-dependent antibody response, from the B cell's CD40 molecule. The ligand for CD40 (CD40L) is a cell surface molecule expressed by activated helper T cells (10). In contrast with the positive effect of receiving two signals, the effect on a B cell of either of the two signals in the absence of the other is negative. Thus, an mIg signal in the absence of a CD40 signal leads either to death of the B cell by apoptosis or to entry into an unresponsive state called anergy (3). By this mechanism, B cells can be tolerized to self antigens not found in the bone marrow, provided no self-antigen-specific T cells are present. In another situation, an irrelevant bystander B cell at the site of an immune response can receive a CD40 signal from incidental contact with an activated T cell. This second signal, in the absence of an antigen receptor signal, also causes apoptosis of the B cell (11). A B cell with relevant mIg specificity survives and makes an antibody response because it receives both an antigen receptor signal and a CD40 signal. Thus, receipt of either signal by itself indicates the absence of an interaction between antigen-specific B and T cells and is deleterious for the B cell. The molecular details of CD40 signaling are currently being unraveled (12). Major questions remain regarding how the individual signaling pathways can trigger apoptosis, as well as what reverses that outcome when the two signaling pathways are combined.

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Germinal center B cells require an antigen receptor signal to survive

During an immune response, antigen-specific B cells migrate to lymph nodes and form germinal centers, where they proliferate rapidly and mutate their immunoglobulin genes at a high rate. The average antibody affinity improves during an immune response ("affinity maturation"), so it appears that there is a mechanism for selecting the higher affinity mutated B cells and removing the lower affinity mutated B cells. It is currently thought that those B cells acquiring antigen receptors with improved affinity for antigen receive an mIg-dependent signal for survival and go on to become memory B cells, whereas those with lower affinity antigen receptors die by apoptosis (13). This process is T cell dependent, and the T cell-derived signal through CD40 also aids B cell survival (14). Interestingly, in the context of a germinal center B cell, mIg contact with antigen promotes survival, whereas in the context of an immature B cell, it promotes elimination. Thus, the differentiation state of the B cell is critical for determining the outcome of mIg engagement

Termination of an immune response: role of the Fc receptor

One mechanism that serves to limit or terminate B cell participation in an immune response is contact of a specific B cell with an immune complex, which is a large matrix of multivalent antigen cross-linked by bivalent antibodies. If the Fc portion of an antibody molecule engages the Fc receptor on the B cell while the antigen engages mIg, as occurs when an immune complex binds to mIg, then antigen receptor signaling is inhibited (15). The molecular basis for this long-observed phenomenon was an enigma until it was recently discovered that the phosphotyrosine phosphatase PTP-1C interacts with the cytoplasmic domain of the Fcγ receptor if it is brought next to the antigen receptor by an immune complex (16). The targets of PTP-1C are

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unknown but could be components of any of the second messenger signaling pathways activated by mIg (see below).

Implementation of the antigen receptor signal: proximal biochemical events

The most proximal events that occur upon antigen receptor signaling have been the best characterized. In order to deliver a signal, mIg must be cross-linked by repeating epitopes on a multivalent antigen. Cross-linking is believed to bring into proximity the components of multiple oligomeric antigen receptor complexes, initiating a cascade of biochemical signaling reactions (17). The degree to which cross-linking occurs correlates with signal strength and with the biological response of the B cell (18). The initial event that is observed upon mIg crosslinking is the phosphorylation on tyrosine residues of a number of proteins in the cell (17). Prominent among these proteins are the antigen receptor components Ig-α and Ig-β. The tyrosine phosphorylation of Ig-α and Ig-β is thought to be mediated by the Src-family tyrosine kinases and this phosphorylation then leads to the binding of another type of tyrosine kinase, Syk, which is important for triggering downstream signalling events (19). The best-characterized of these events are the Ras-MAP kinase cascade of protein phosphorylation (20, 21), the phosphoinositide pathway (22), which is activated by the tyrosine phosphorylation of phospholipase Cγ2, and the activation of PI-3 kinase (23, 24), which may activate protein kinase C-ε (25). The transmembrane tyrosine phosphatase CD45 has been shown to be required for antigen receptor signaling, possibly owing to its ability to dephosphorylate a negative regulatory tyrosine found in all Src family kinases (26). In addition to the most proximal events that are directly connected to the antigen receptor, many of the signaling pathways and components activated by the mIg

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signaling are common to other receptor systems, including others of the B cell itself. It is not known how the particular combination of signaling events used results in antigen receptor-specific signals for the cell or how the antigen receptor signal is modified to cause the different biological outcomes described above.

Regulation of early response gene expression by antigen receptor signaling

Most of the mIg-induced biochemical changes, such as in the protein phosphorylations or in the abundance of the phosphoinositide metabolites, are transient, reaching their maximum amplitude within minutes and reverting to nearly unstimulated conditions in the subsequent minutes to hours. It has been proposed that biological changes in the cell that occur over longer periods of time are carried out by changes in the abundance of cellular components. Alterations in the abundance of proteins can occur through changes in gene expression—namely in the rates of synthesis or degradation of the proteins or their mRNAs or both. Early response genes have been defined as those genes whose mRNA levels change upon receptor signaling owing to the action of pre-existing components—for example, in the absence of the synthesis of any intervening factors (27). Early response genes were first identified in quiescent fibroblasts that were stimulated with serum-derived growth factors, which cause entry into the cell cycle. The translation inhibitor cycloheximide was used to determine which inductions depended solely on pre-existing proteins. Coincidentally, this strategy provided the additional benefit to researchers of "superinducing" the early response gene mRNAs, presumably by preventing synthesis of labile repressive factors or factors that carry out the degradation of certain mRNAs. Like many of the biochemical signaling events described above, early response gene induction

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is rapid and transient. Many of the early response genes identified have turned out to be either nuclear oncogenes or signaling molecules, implying that the products of these genes play important roles in signaling for growth regulation. Among the most common types of early response genes found were transcription factors, which were hypothesized to regulate expression of a set of secondary genes, whose expression may be more closely tied to long-term effects. Investigation of the secondary, or delayed early, response genes has only recently been undertaken (28, 29).

Because the early response genes are likely to represent key intermediaries, or "third messengers," between the biochemical signaling pathways and the secondary response genes, I was interested in identifying those early response genes that are induced through the antigen receptor of B lymphocytes. I intended to use the early response genes not only as readouts for the mIg-mediated activation of upstream second messenger pathways, but also as possible means of identifying downstream events that specify the outcomes of B cell stage-specific cellular responses.

Identification of B cell antigen receptor-induced early response genes
A B cell response that is readily studied in the context of a tissue culture system is the apoptotic response to mIg signaling. WEHI-231 is a B lymphoma-derived cell line whose growth is arrested upon mIg cross-linking, subsequently undergoing apoptosis (30, 31). This cell line is a widely studied model for the antigen receptor-mediated clonal deletion of immature B cells. The proximal biochemical events induced by antigen receptor signaling in WEHI-231 cells are very similar to those of normal B cells. I set out to identify the mIg-induced early response genes in WEHI-231 cells by

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differentially screening a subtracted cDNA library that was enriched for mRNAs induced by two hours of treatment with anti-IgM antibodies and cycloheximide. Identified in this screen were three early response genes, *egr-1, jun-B,* and *nup475,* that had previously been identified in serum-stimulated fibroblasts (32). Two of the three (*egr-1* and *jun-B*) had also been recently reported to be activated by mIg in B cells (33, 34). As an alternative approach to characterizing mIg-induced early response genes, I tested a set of seven of the major genes induced by serum in fibroblasts (32) and found four of them (*pip92, nup475, 3CH134,* and *nur77*) to be induced in normal splenic B cells. Some of these genes were differentially induced between B cells and two B lymphoma-derived cell lines. These studies are discussed in the first chapter of the thesis.

Approaches to understanding the roles of early response gene inductions

The early response gene literature has until recently been largely descriptive. Because early response genes have been found to encode signaling molecules such as transcriptional regulators, cytokines, and phosphatases, it has been surmised that they will turn out to play important roles in cellular responses to stimuli. Little is known about whether the inductions are gratuitous or are in fact required for biological responses, and events immediate downstream of early response gene induction have generally not been defined. The question of necessity can be addressed through inhibiting the expression or the function of the gene in question. Available strategies for inhibiting gene expression include the addition to cells of antisense oligonucleotides, the expression of antisense mRNA constructs, or knockout of targeted genes by homologous recombination. Alternatively, the functions of the early response gene protein products can be inhibited by introducing into cell lines

dominant negative mutant alleles, either of the genes in question or of genes known to interact with them. The best candidates for the alternative type of approach are the transcription factors. A transcription factor typically comprises separable domains that mediate transactivation, dimerization, and DNA-binding, allowing for the design of dominant negative alleles that will disrupt one of these functions. In addition, the target DNA regulatory sequences of the transcriptional regulators can be screened for, as can the target genes themselves. As an alternative approach, overexpressing an early response gene product can be informative, either alone or in conjunction with the intact receptor signal.

C-*myc* is the prototype early response gene

The proto-oncogene c-*myc* was shown to be induced in response to mitogen stimulation in B and T lymphocytes and in fibroblasts, and as such was the first gene to be described as an early response gene (35). C-*myc* was later shown to be induced in B cells by antigen receptor stimulation as well (36). Although c-*myc* is a widely expressed gene and behaves as an early response gene in response to many growth-related stimuli, there is a particular connection between c-*myc* and the B cell. Among the most prominent examples of tumors in which c-*myc* has been implicated are avian leukosis virus-induced bursal lymphomas (37), murine plasmacytomas, and human Burkitt lymphomas (38), all of B cell origin.

The function of c-Myc appears to be related primarily to promotion of cell proliferation. The oncogenic property of c-*myc* derives simply from the overexpression of its product, rather than from any alteration in function that would arise from mutation of the protein itself. Similarly, the

overexpression of an inducible form of c-Myc can drive serum-starved fibroblasts to enter the cell cycle (39). Moreover, in nontransformed cells, a correlation has been noted between the levels of c-*myc* expression in a cell and the rate at which that cell proliferates. These observations have led to the hypothesis that c-*myc* is intimately involved in the cell cycle and the level of c-Myc plays an important role in determining the rate of proliferation of a given cell (35, 40, 41).

More recently, it was discovered by Evan et al. that the forced expression of c-Myc in serum-starved fibroblasts causes apoptosis as well as proliferation. Thus c-*myc* became implicated in apoptosis as well as cell cycle progression (42). C-*myc* has also been implicated in the antigen receptor-stimulated apoptosis of T cell hybridomas through the use of dominant negative proteins in a transient transfection assay (43). In addition, deprivation of IL-3 from an IL-3-dependent cell line overexpressing c-Myc leads to apoptosis (44). Thus, c-*myc* appears to play an important role in the apoptosis of numerous cell types.

The possible role of c-*myc* in the antigen receptor-mediated apoptosis of B cells

The second chapter of this thesis describes my investigation into the possibility that c-*myc* plays a role in the antigen receptor-mediated apoptosis of WEHI-231 cells. A role for c-*myc* in mIg-induced apoptosis of these cells was first suggested by the work of Fischer et al. (45), who used an antisense oligonucleotide to inhibit c-*myc* expression. This result may not be meaningful, however, because the oligonucleotide used contained a short CG-based sequence that has potent B cell stimulatory properties. Thus,

inhibition of apoptosis seen in those experiments may have been caused by a mechanism unrelated to inhibition of c-*myc* expression (46). To study the involvement of c-*myc* in B cell apoptosis further, I initially asked whether the induction of Myc would be sufficient to bring about growth arrest and apoptosis. Therefore, the overall amount of Myc in WEHI-231 cells was increased by introducing a hormone-regulatable chimeric form of Myc (MycER). I found that activating MycER induced apoptosis directly and accelerated the rate of mIg-induced apoptosis. Although these observations were consistent with c-*myc*'s participation in antigen receptor-mediated apoptosis, other observations indicated that the antigen receptor signal was not solely mediated by the elevation of c-Myc levels and must rely on other pathways as well.

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Chapter 1.

Induction of Early Response Genes by Cross-linking Membrane Ig
on B Lymphocytes

ABSTRACT

Cross-linking of membrane Ig (mIg) on B lymphocytes induces protein
tyrosine phosphorylation and phosphoinositide hydrolysis, events which are
thought to mediate the diverse biological responses of B cells to antigen
binding. mIg stimulation also induces the expression of the putative
transcriptional regulators c-*myc*, c-*fos*, *egr-1*, and *jun-B*. In this report, normal
murine B cells and two murine B lymphoma cell lines were examined for the
induction of mRNA expression of seven early response genes first identified
in fibroblasts. Expression of four of the seven genes (*nur77, nup475, pip92*
and *3CH134*), encoding two putative transcriptional regulators and two
proteins of unknown function, was induced following cross-linking of mIg in
resting B cells isolated from mouse spleen. In the 2PK-3 and WEHI-231 B
lymphoma cell lines three and two, respectively, of these four genes were
induced. Expression of these genes could be induced in 2PK-3 cells by
activating the phosphoinositide signaling pathway independently of the
tyrosine phosphorylation pathway by signaling through an M1 muscarinic
acetylcholine receptor introduced by transfection. Additionally, in all but one
case, these early-response genes could be induced by directly activating protein
kinase C (PKC) with phorbol esters. In the cell line 2PK-3, the gene *3CH134*
was not induced by phorbol ester treatment, but was induced by elevation of
intracellular calcium. Thus, a subset of the early-response genes identified in
serum-stimulated fibroblasts is also induced by antigen receptor stimulation
in B lymphocytes, and this induction appears to be mediated by the
phosphoinositide signaling pathway and, for the most part, protein kinase C.

INTRODUCTION

B cells recognize the presence of antigens in the extracellular milieu by means of immunoglobulin antigen receptors expressed on the cell surface. Cross-linking of membrane immunoglobulin (mIg) by multivalent antigen initiates important changes in the physiological state of the B cell. Cross-linking of mIg on an immature B cell can induce unresponsiveness (clonal anergy) (1) or programmed cell death (clonal deletion) (2), processes which are thought to be important for removing self-reactive B cells (3). In contrast, the same treatment of a mature B cell stimulates entry into the active cell cycle, proliferation and responsiveness to cytokines that promote antibody production and secretion (4). Thus, the B cell antigen receptor plays a critical role in making appropriate and effective immune responses.

Much has been learned recently about the biochemical reactions that take place after receptor cross-linking. The initial signaling event is now thought to be the induction of protein tyrosine phosphorylation (5-8). Targets of tyrosine phosphorylation include several tyrosine protein kinases (9-11), the mIg-associated proteins Ig-α and Ig-β (12), the GTPase-activating protein, GAP (13), and phosphatidylinositol 3-kinase (14, 15), as well as phospholipase Cγ1 and Cγ2 (16-20), enzymes that can hydrolyze phosphotidylinositol 4,5-bisphosphate (PIP$_2$) and initiate the phosphoinositide signaling pathway. Among these events, phosphoinositide breakdown has received considerable attention as a key signaling event in both mature and immature B cells. Although PIP$_2$ breakdown appears to be an important signaling event in mediating the mIg-induced G$_0$ to G$_1$ transition in mature B cells (21-23) and the growth arrest of the immature B lymphoma cell line WEHI-231 (24),

recent evidence suggests that immature B cells from neonatal murine spleen fail to induce PIP_2 breakdown in response to mIg cross-linking (25). Thus, PIP_2 breakdown may not always be stimulated by mIg cross-linking and other signaling reactions may be important for mediating some of the B cell's responses to antigen.

Both the stimulation of mature B cells with anti-Ig and the addition of serum to quiescent fibroblasts in culture stimulates the transition from the resting G_0 state into the G_1 phase of the cell cycle. Serum stimulation of fibroblasts also induces mRNA expression of a large number of genes called early response genes (26, 27). The protein products encoded by many of these genes are thought to play important roles in carrying out the genetic program required for receptor-mediated cellular processes. This view is supported by the fact that many of the products of early response genes are known or putative transcriptional regulators. These gene products may be classified as "third messengers" because of their generality to many receptors that induce distinct outcomes with regard to cell proliferation or differentiation (28, 29). In B cells, the early-response genes c-*myc*, c-*fos*, *egr-1*, and *jun-B* (30-33) have thus far been reported to be activated upon stimulation of the antigen receptor.

As a further step in defining the set of genetic changes initially responsible for implementing instructions from the antigen receptors in mature and immature B cells, we examined the ability of mIg to induce expression of a panel of early response genes originally described in murine fibroblasts (26). Encoded by these seven genes are three nuclear proteins that may be transcription factors and four proteins of unknown function. Four of these

genes, two of which may be transcription factors, were found to be expressed after cross-linking of mIg on B cells. Additionally, in an initial effort toward describing the sequence of events leading to induction of these genes, we examined the second messenger pathways responsible for their activation.

MATERIALS AND METHODS

Cells and cell culture Mouse B lymphoma cell lines WEHI-231 and 2PK-3 were cultured at 37°C in an atmosphere containing 5% CO_2 in RPMI 1640 medium (M.A. Bioproducts, Walkerville, MD) supplemented with 10% heat-inactivated FCS (Hyclone, Logan UT), 1 mM sodium pyruvate, 2 mM glutamine and 50 mM 2-mercaptoethanol. Cell lines were grown in logarithmic phase at cell densites of less than 5 x 10^5/ml. Splenic resting B cells were prepared as described (5). Briefly, spleen cells were depleted of T cells by two sequential treatments with anti-CD4, anti-CD8, and anti-Thy-1 antibodies plus guinea pig complement. Resting B cells were isolated on a Percoll density gradient and suspended in culture medium (see above). Cells were stimulated directly after isolation, because murine B cells do not survive well in culture in the absence of stimulation. Stimulation of normal B cells was performed in a 37°C incubator (see above) at 5-10 x 10^6 cells/ml. Cell lines were stimulated in a 37°C water bath at cell densities of 1-4 x 10^6 cells/ml in medium buffered with 20 mM Hepes, pH 7.4.

Reagents Goat anti-IgM and goat anti-IgG, Fc-specific (Jackson Immunoresearch Laboratories, Westgrove, PA), antibodies were used at 10-20 µg/ml. Stock solutions of various agents were as follows: PdBu (Sigma Chemical Co., St. Louis, MO) was dissolved at 200 µM in ethanol; ionomycin (Calbiochem, La Jolla, CA) was dissolved at 1.7 mM in DMSO; cycloheximide (Sigma) was dissolved at 10 mg/ml in water; carbamylcholine chloride (carbachol, Sigma) was dissolved at 250 mM in water; atropine (Sigma) was dissolved at 25 mM in 25% methanol/75% H_2O; compound 3 was a gift from

Dr. M. Venuti, Department of Bio-Organic Chemistry, Genentech (SanFrancisco, CA), and was dissolved at 10 mM in ethanol.

Transfections The plasmid containing the human muscarinic receptor type 1 (HM1) gene, pHM1-SFNeo, was obtained from Dr. A. Weiss (UCSF) (34). This construct was introduced into 2PK-3 cells by electroporation, using a "Gene Pulser" (Bio-Rad Laboratories, Richmond, CA) at settings of 300 V and 250 mF. Stable transfectants were selected and maintained in 10% FCS supplemented with 0.5 mg/ml G418. Receptor expression was measured by Scatchard analysis using specific binding of ^{3}H-labeled quinuclidinyl benzilate ([^{3}H]-QNB, 42 Ci/mmol, Amersham, Arlington Heights, IL) at room temperature to measure the total number of HM1 receptors expressed per cell (35).

RNA isolation and northern blot analysis Total RNA was extracted from cells by the acid phenol method (36) with the exception that only a single isopropanol precipitation was performed. RNA (2.0-2.5 µg/lane) was resolved by electrophoresis through 1.2% agarose gels containing 2 M formaldehyde, buffered with 5 mM Hepes, pH 7.0, transferred to "Genescreen" membranes (NEN Research Products, Boston, MA), and crosslinked to the membranes by UV irradiation by a Stratalinker 1800 (Stratagene, La Jolla, CA). All experiments were conducted with at least two independent preparations of RNA, with the exception of the compound 3-treated 2PK3-HM1 cells stimulated with carbachol (Fig. 5B). Specific RNA was detected by probing the membranes with random oligonucleotide-primed (Boehringer Mannheim Biochemicals, Indianapolis, IN) ^{32}P-labeled cDNA probes. Hybridization was performed in buffer containing 50% formamide, 1 M NaCl, 1% SDS, 10%

dextran sulfate, and 125 µg/ml denatured salmon sperm DNA. Filters were washed in 0.1 X SSC and 1% SDS at 65°C to remove nonspecific binding of the probe. To strip off probe for subsequent reprobing, blots were boiled for 10 minutes in 0.1% SDS. To determine that equal amounts of mRNA were loaded in each lane, the stripped blots were reprobed with the cDNA of the noninduced, constitutively-expressed gene GAPDH (37). This control was performed with each blot incorporated into the figures, and was found to be satisfactory. Densitometric analysis using a Joyce Loebl densitometer (Vickers Instruments, Malden, MA) indicated that almost every blot had variations of GAPDH hybridization of +/- 30% or less among lanes and in the few exceptions the variation in GAPDH was minor compared to the effects of the experimental treatments. All observations were made with at least two independent northern blots and typically with many more.

cDNA probes Probes for northern blot analysis were made from Eco R1 fragments of the respective Balb/c mouse cDNAs of seven fibroblast early-response genes (cyr61, nur77, pip92, 3CH134, HLH462, nup475, and 3CH482), which were obtained from Dr D. Nathans (Johns Hopkins) (26).

Western blots Western blots were performed as described (5). Triton X-100-soluble cell lysates made in buffer containing 1 mM sodium orthovanadate and protease inhibitors were separated on 10% SDS-PAGE gels, probed with the phosphotyrosine-specific monoclonal antibody 4G10 (38), and detected with an alkaline phosphatase-anti-mouse IgG conjugate (Bio-Rad) and 5-bromo-4-chloro-3-indoyl phosphate/nitro blue tetrazolium (BCIP/NBT). Prestained m. w. markers were from BioRad.

RESULTS

Four early-response genes are induced in B lymphocytes

A set of seven fibroblast-derived early response genes (26), not previously reported to be expressed in B cells, were tested for induction of mRNA expression upon signaling through mIg. Small, dense, resting B cells and two B lymphoma-derived cell lines, WEHI-231, an IgM-expressing immature B cell (39) and 2PK-3, an IgG-expressing memory B cell (39), were treated with anti-Ig antibodies to trigger mIg signaling, and total cellular RNA was isolated after various times. Four of the seven fibroblast early response genes were induced in the splenic B cells after anti-Ig stimulation. Northern blot analysis of RNA from the three types of B cells revealed a distinct pattern of expression and induction for each of the four early response genes. The early response gene *pip92* was found to be induced approximately 5-10-fold in all three types of B cells examined (Fig. 1). The gene *nup475* was strongly induced in WEHI-231 cells and 2PK-3 cells (roughly 5-fold), and induced less well in B cells (~2-fold). The gene *3CH134* was found to be induced in 2PK-3 cells (about 4-fold), minimally induced in B cells (2-fold) (Fig. 1), but not detected in WEHI-231 cells. The gene *nur77* was found to be expressed only in B cells, where it was strongly induced (~fivefold). Blots containing the three cell types together show the relative expression and magnitude of induction of the three genes expressed in more than one cell type (Fig. 2). In general, the level of induced expression attained was highest in normal B cells, intermediate in 2PK-3 cells, and lowest in WEHI-231 cells. The baseline expression of these genes was also somewhat higher in B cells than in the cell lines. We do not think that this basal expression was due to the occurance of a low level of activation in the course of cell purification or *in vivo* prior to

FIGURE 1. Induction of early response gene messenger RNA expression in normal B cells and B- lymphoma-derived cell lines. Total RNA (2.0 μg for B cells and 2.5 μg for the cell lines WEHI-231 and 2PK-3) isolated from cells at different times after treatment with anti-Ig (in the presence or absence of CHX) was subjected to Northern blot analysis. Filters were probed with the indicated genes and were subsequently stripped and reprobed with GAPDH to verify equivalent loading of mRNA in each lane (not shown). Cells were treated for the indicated times with the following reagents: 10 μg/ml anti-IgM (B cells and WEHI-231) or anti-IgG (2PK-3), and 10 μg/ml cycloheximide (added concurrently with anti-Ig) as indicated. Where no mRNA was detected in a particular cell line, the data are not shown.
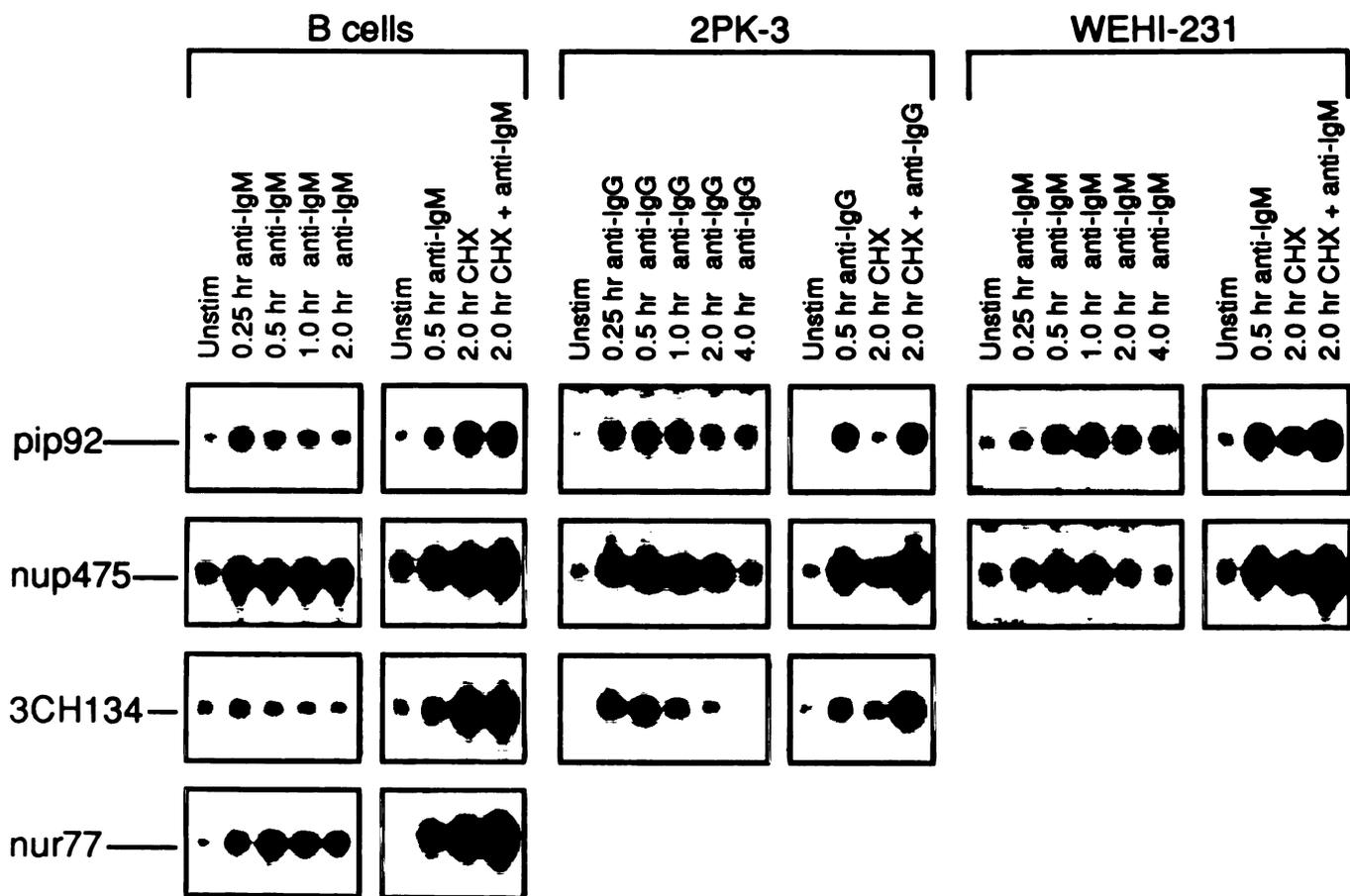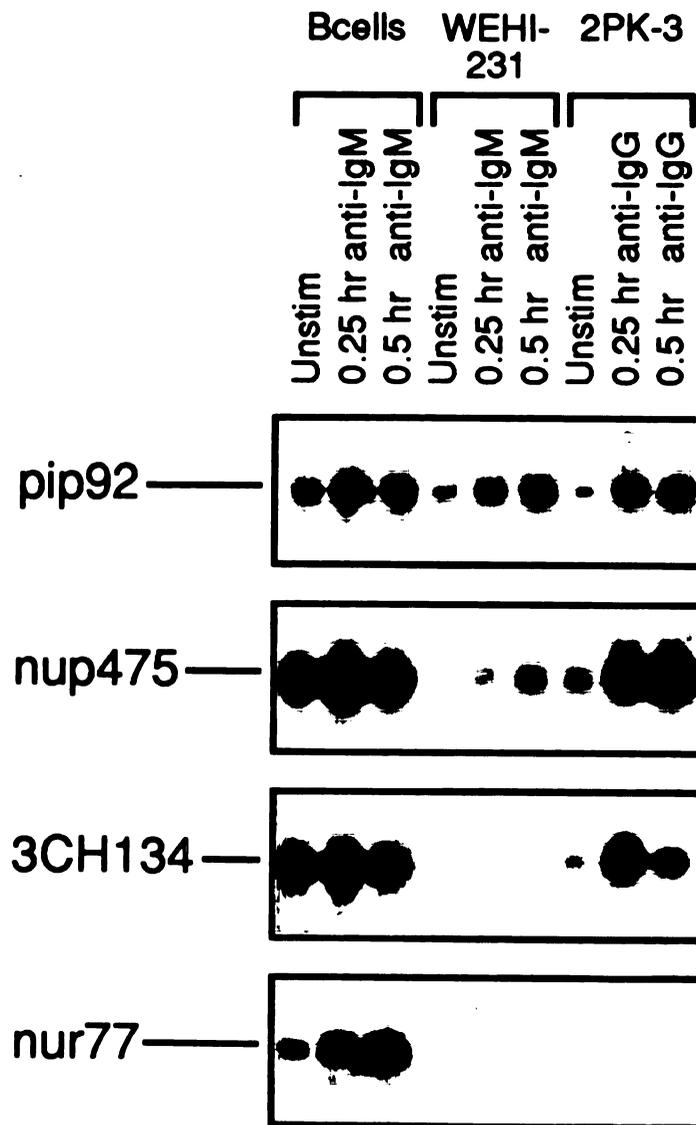
FIGURE 2. Relative amounts of early response gene expression in different B cells stimulated with anti-Ig. Total RNA (2.0 µg for B cells and 2.5 µg for the cell lines WEHI-231 and 2PK-3) from each cell type, unstimulated or stimulated with anti-Ig for the indicated times, were run together on the same gel and subjected to Northern blot analysis as in Figure 1.

isolation, because the same blots, stripped and reprobed with c-*fos*, indicated

that c-*fos* expression was minimal in unstimulated B cells (data not shown),

in accord with earlier reports (31, 40). Among the uninduced genes, the gene

HLH462 was expressed constitutively both in B cells and in the lymphoma-

derived cell lines, and the other two early-response genes tested, *cyr61* and

3CH482, were detected neither before nor after stimulation (data not shown).

cyr61 and *3CH482* were also not detected when CHX (see below) was included

with anti-Ig. We also found that c-*jun*, which is induced by serum in

quiescent fibroblasts, was only induced in the cell lines when CHX was

included with anti-Ig, or in normal B cells when phorbol and ionomycin

were added together or CHX was added alone (data not shown). Thus, a

subset of the early response genes induced in fibroblasts by serum growth

factors is induced in B cells by cross-linking the antigen receptor.

The protein synthesis inhibitor CHX did not block the induction of these

genes by mIg cross-linking (Fig. 1). The induction of these genes in B cells can

therefore be described as an immediate response in that it depended solely

upon the action of pre-existing proteins. Furthermore, the kinetics of the

induction of each of the four genes corresponded to that of other early

response genes in that it was rapid and transient: maximal mRNA induction

occured by approximately 30 minutes, and message levels declined within the

next several hours (Fig. 1). Also characteristic of early-response gene

induction is its greater magnitude and longer duration when CHX is present

along with the inducing agent. This phenomenon, termed superinduction, is

attributed to the blocking of two processes presumed to be dependent on labile

proteins: transcriptional repression or degradation of these unstable mRNAs

or both (41). As can be seen in Figure 1, CHX enhanced and prolonged the

induction by anti-Ig of all four of these early response genes. In most cases, CHX treatment by itself increased message levels of these genes to some extent, indicating that the baseline mRNA levels were controlled in part by one or both of these processes.

Heterologous activation of the phosphoinositide pathway induces early-response genes

Our next step was to explore the mechanism by which mIg cross-linking activated these early response genes. Anti-Ig antibodies induce rapid tyrosine phosphorylation of a variety of targets, as well as phosphoinositide breakdown. Tyrosine kinase inhibitors block both of these signaling reactions, suggesting that tyrosine phosphorylation is upstream of phospholipase C (PLC) activation (7, 16, 42). Indeed, among the targets of anti Ig-induced tyrosine phosphorylation are PLC-γ1 and PLC-γ2 (16-20). In any case, the resulting phosphoinositide breakdown is a prominent signaling event in B cells undergoing Ag-receptor stimulation. Thus, we decided to test whether PIP_2 breakdown played an important role in activating mRNA expression of the early response genes. Our first approach toward addressing this question was to stimulate PIP_2 breakdown by a mechanism that is not mediated by protein tyrosine phosphorylation. For this purpose, we used the human muscarinic acetylcholine receptor type 1 (HM1). This receptor is a protein with seven membrane-spanning domains that activates PLC by means of a G protein that is insensitive to pertussis toxin (43) (possibly employing PLC-β by means of G_q) (44, 45) and thus affords an alternative means of activating the phosphoinositide pathway in a physiological manner. An expression vector coding for the HM1 (34) was transfected into the 2PK-3 cell line. Scatchard analysis of a radiolabeled ligand bound to the cells
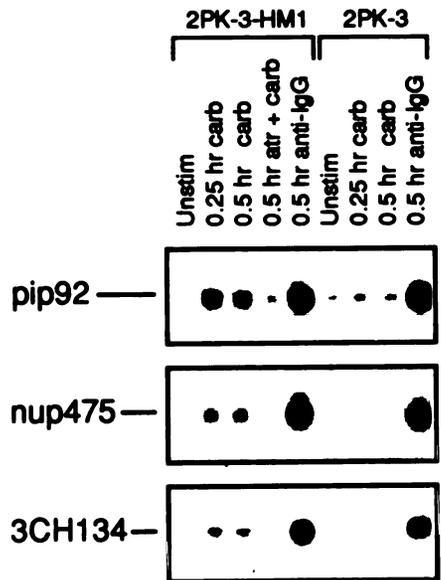
FIGURE 3. Induction of early response genes by human muscarinic acetylcholine receptor in transfected 2PK-3 cells. 2PK-3 cells expressing transfected human muscarinic acetylcholine receptor type 1 were cultured at 4 x 10^6 cells/ml and treated for the indicated times with the following reagents: 1mM carbachol (agonist), 0.5 mM atropine (antagonist), or 20 μg/ml anti-IgG as indicated. Northern blot analysis was performed as in Figure 1.

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revealed that approximately 40,000 HM1 receptors were expressed in several transfectants (data not shown). Addition of the agonist carbachol to these transfectants clearly induced expression of two of the three early response genes found to be induced by mIg cross-linking in 2PK-3, *pip92* and *nup475* (Fig. 3). Expression of the gene *3CH134* was increased by carbachol to a lesser extent. The induction in response to carbachol exhibited similar kinetics to that of the induction due to mIg cross-linking. The levels of mRNA expression induced by carbachol were lower than those induced by anti-IgG. This could implicate an intracellular pathway in addition to PIP2 breakdown in the mIg-derived signal. Other explanations include the possibility that the HM1 receptor was less effective at causing prolonged PIP2 hydrolysis—for example owing to receptor desensitization (46) or to an insufficient number of HM1 receptors, G proteins, or PLC-β molecules. In any case, these experiments suggest that the phosphoinositide signaling pathway plays an important role in the induction of early response genes by mIg.

Role of protein kinase C in the induction of early-response genes by mIg The hydrolysis of PIP2 by PLC leads to the activation of two second messenger pathways: an increase in intracellular calcium and the activation of protein kinase C (PKC). These two second messenger pathways constitute the major known effects of PIP2 breakdown and are believed to account for the biological effects of receptor signaling in many cases (47, 48). To dissect the second-messenger pathways that play a role in activating the early-response genes, we next used two pharmacologic agents that mimic the effects of phosphoinositide breakdown: ionomycin, a calcium ionophore which elevates intracellular free calcium; and phorbol esters, which activate PKC. Cells were treated either with ionomycin or with (PdBu) or with both

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concomitantly, at concentrations that closely reproduce the levels of intracellular free calcium and PKC activation seen after anti-Ig treatment of WEHI-231 cells (250 nM and 7 nM respectively) (24) (D. M. Page, PhD thesis, University of California at San Francisco, San Francisco, CA). The early response genes *pip92* and *nup475* were induced in the cell lines by PdBu alone to an extent that approximated the induction by anti-Ig (Fig. 4). All four of the genes were induced in normal B cells by PdBu and curiously to a much greater extent than by anti-IgM. It would appear that 7 nM PdBu activates PKC in splenic B cells to a greater extent or in a more prolonged fashion than does mIg signaling. Ionomycin did not clearly augment the effect of PdBu in any case except for the induction of *3CH134* in 2PK-3 cells. For this gene, induction in response to ionomycin alone was observed, and was nearly comparable to the induction in response to anti-IgG. PdBu alone had no effect on *3CH134* expression in 2PK-3 cells and did not enhance the response to ionomycin. Thus, *3CH134* is unusual among early response genes in that under some circumstances (e.g. in 2PK-3 cells) it is not inducible by phorbol esters. Although ionomycin alone did not induce *3CH134* expression in splenic B cells, it did induce c-*fos* slightly in these experiments when the blots containing splenic B-cell RNAs were stripped and reprobed with c-*fos* (data not shown). This is in agreement with previously reported results (40) and demonstrates that the ionomycin stimulation was effective in the splenic B cells. As the mRNAs for *pip92, 3CH134, nup475* and *nur77* were induced by phorbol ester treatment in splenic B cells, it appears that the activation of PKC can directly cause the induction of these four early-response genes. These observations are consistent with the hypothesis that PKC mediated the mIg-directed induction of these early response genes, with the one exception described above.

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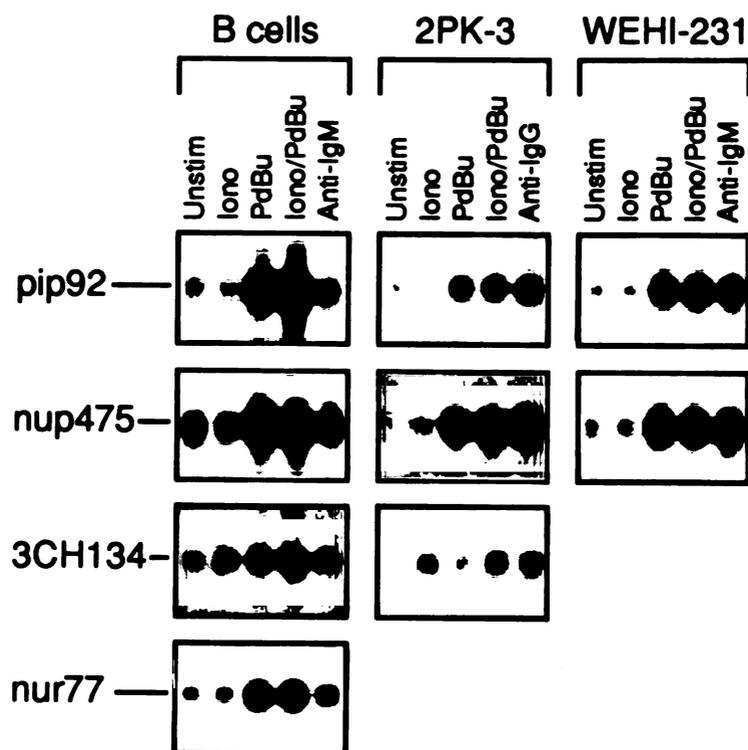
FIGURE 4. Induction of early response genes by pharmacologic activators of phosphoinositide signaling events. Cells were treated for 0.5 hr with the following reagents as indicated: 250 nM ionomycin, 7 nM PdBu, the combination of 250 nM ionomycin and 7 nM PdBu, or 10 mg/ml anti-Ig. Total RNA was prepared from cells and subjected to Northern blot analysis as in Figure 1.

Another approach to exploring the role of PKC in the induction of early response genes by mIg is to assess the effects of PKC inhibitors. In our experience, the staurosporine analog compound 3 (49) appears to be an effective inhibitor of PKC without having excessive toxicity (50, 51). When WEHI-231 and 2PK-3 cells were pretreated for 20 minutes with various concentrations of compound 3, the anti-Ig- and PdBu-mediated inductions of *pip92, 3CH134,* and *nup475* were blocked in a similar and dose-dependent fashion (Fig. 5A). Compound 3 (10 μM) completely blocked induction of each gene. 3 μM compound 3 also blocked the ionomycin- and anti-IgG-mediated induction of *3CH134* in 2PK-3 cells (Fig. 5A). No effect of compound 3 was seen on the mRNA levels of the constitutively expressed gene that encodes *GAPDH* (data not shown). Interestingly, the baseline expression of each of the early-response genes was reduced by compound 3. Thus, the baseline expression of the early response genes as well as the intracellular calcium-mediated induction of *3CH134* may have required a low level of PKC activity. Compound 3 also blocked the carbachol-mediated gene induction of *pip92* and *nup475* in HM1-transfected 2PK-3 cells (Fig. 5B), indicating that the HM1 receptor also required PKC to induce expression of these early response genes. In Balb/c 3T3 fibroblasts, the induction of c-*fos* by serum was only partially inhibited by 1 μM compound 3, whereas the induction by PdBu was totally blocked (data not shown). This observation is consistent with the conclusion reached by others that some early-response genes are activated by a PKC-independent pathway in fibroblasts (52-54), and indicates that compound 3 does not block early-response gene induction under all circumstances.

Treatment of B cells and B cell lines with anti-Ig antibodies causes the phosphorylation on tyrosine of several protein species as detected by

FIGURE 5. Effect of a PKC inhibitor on the induction of early response genes by means of membrane immunoglobulin or the human muscarinic acetylcholine receptor. Total RNA was prepared from cells and subjected to Northern blot analysis as in Figure 1. A.) 2PK-3 or WEHI-231 cells were incubated at a density of 4×10^6/ml in 20 mM Hepes buffered media and pre-treated with compound 3 at the concentrations indicated for 20 minutes. Cells were subsequently treated for 15 minutes with 10nM PdBu or 20 µg/ml anti-Ig in the continued presence of compound 3. B.) 2PK-3 cells expressing the transfected human muscarinic acetylcholine receptor (2PK-3-HM1) were pretreated as indicated for 20 minutes with 10 µM compound 3. Cells were subsequently treated with: 1 mM carbachol (from 3 to 15 minutes, as indicated) or with 10 nM PdBu or 20 µg/ml anti-IgG for 15 minutes.
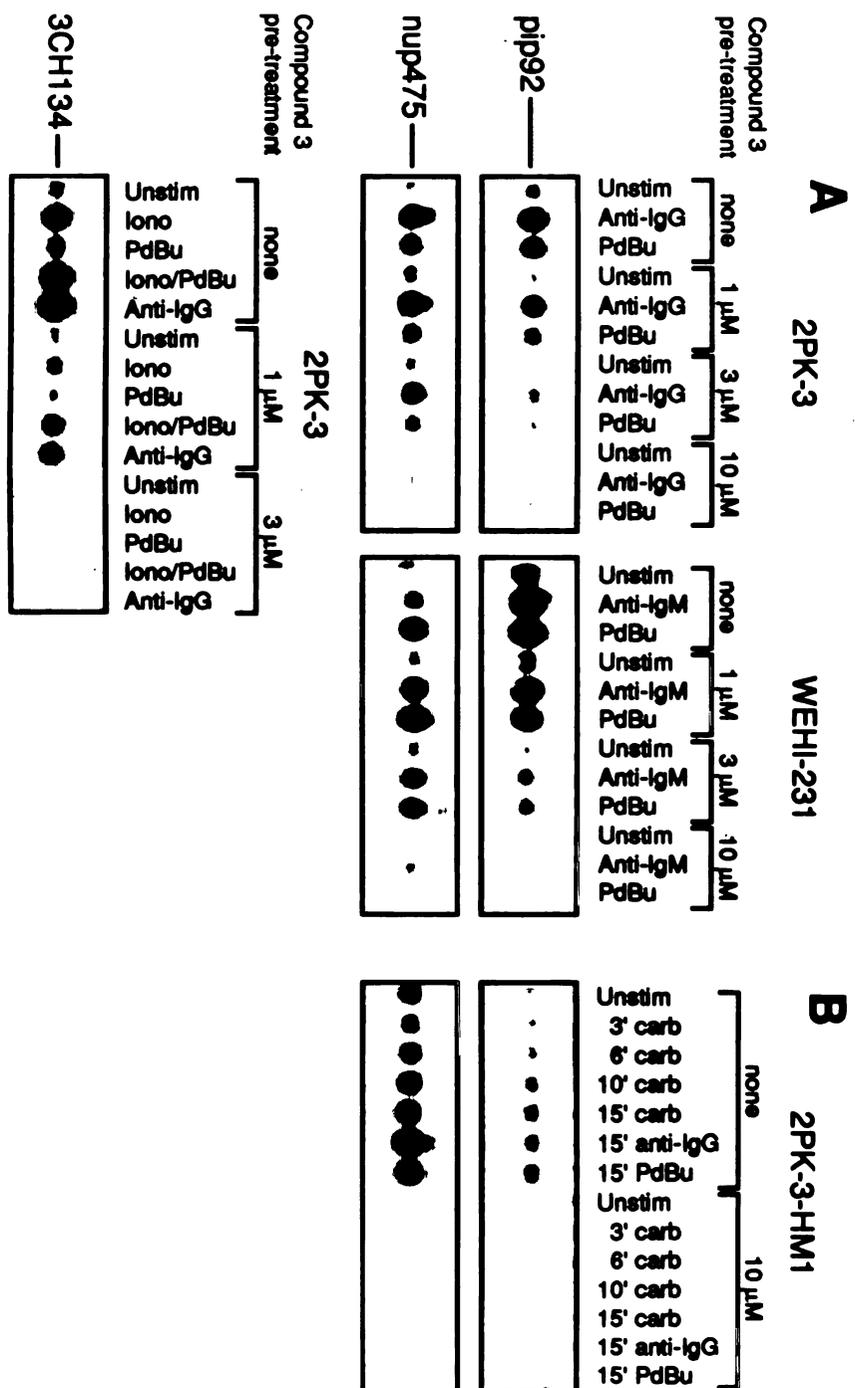
antiphosphotyrosine Western blotting (5, 6). Of these, the only major protein tyrosine phosphorylation that appears following treatment of these cells with phorbol esters is the species migrating at ~42 kD (5). This substrate is probably MAP kinase as this enzyme is activated and tyrosine phosphorylated in response to anti-Ig or phorbol esters (51, 55). The tyrosine phosphorylation of MAP kinase is thought to be an indirect effect of the activation of PKC because the latter enzyme phosphorylates proteins only on serine and threonine residues. As expected, stimulation of the 2PK-3 cells expressing the HM1 receptor with anti-IgG caused tyrosine phosphorylation of many proteins (Fig. 6). Stimulation of these cells with carbachol induced tyrosine phosphorylation of only the 42 kDa MAP kinase band. Strong tyrosine phosphorylation of this protein was also seen after treatment with PdBu. Compound 3 pretreatment blocked the PdBu-induced tyrosine phosphorylation of this band. Among the species tyrosine phosphorylated in response to anti-Ig, compound 3 inhibited the appearance of only the 42 kDa band (Fig. 6). A band also appeared migrating at ~75 kDa, when cells treated with compound 3 were stimulated by any of the agents that activate PKC. We do not know the identity of this species, whose induced tyrosine phosphorylation appears to be inhibited by PKC. Taken together, these results suggest that compound 3 blocked the action of PKC but not the mIg-activated tyrosine kinases. As the tyrosine phosphorylation of the MAP kinase band in response to anti-IgG or carbachol was clearly blocked less well than the response to PdBu, these receptors may have induced the tyrosine phosphorylation of this protein by two routes, only one of which was PKC-dependent. Alternatively, the several isozymes of PKC expressed in B cells (56) may be differentially subject to inhibition by compound 3. In any case the PKC isozymes that are sensitive to compound 3 appear to include those

isozymes that play a role in induction of the early response genes *pip92*, *3CH134*, and *nup475* in B cell lines.

Although MAP kinase has been suggested to play a role in the activation of transcription factors (57), we did not observe a strong correlation between tyrosine phosphorylation of the protein likely to be MAP kinase and the expression of early response genes. At lower concentrations of PdBu, the magnitude of induction of early response genes in 2PK-3 was approximately proportional to the magnitude of increase in tyrosine phosphorylation on the 42 kDa protein (data not shown). In contrast, compound 3 did not block phosphorylation of the 42 kDa protein following carbachol stimulation of the HM1 receptor in the 2PK-3-HM1 cells (Fig.6), whereas the induction of early response genes was blocked (Fig.5). Thus it seems that activation of early response genes induced by the HM1 receptor was not solely a consequence of MAP kinase activation. In any case, the ability of compound 3 to completely block the increased expression of the early-response genes caused by anti-Ig treatment provides additional evidence to suggest that a PKC isozyme mediated the signal transduced by mIg.

FIGURE 6. Effect of a PKC inhibitor on tyrosine phosphorylation induced by phorbol dibutyrate, anti-Ig or carbachol. Portions of the 2PK-3-HM1 cells described in Figure 5B were removed for preparation of total cellular protein lysates which were subjected to Western blot analysis with a phosphotyrosine-specific mAb.
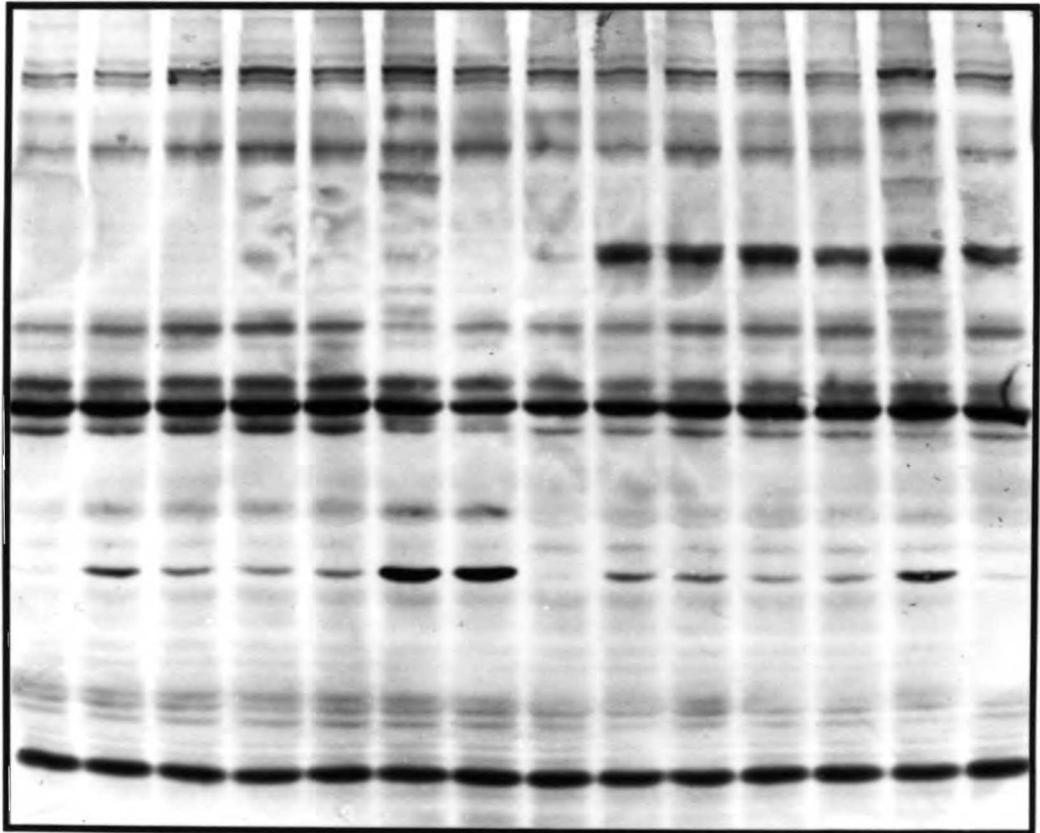
2PK3-HM1

pre-treatment: none   10 µM compound 3

Unstim
3' carb
6' carb
10' carb
15' carb
15' anti-IgG
15' PdBu
Unstim
3' carb
6' carb
10' carb
15' carb
15' anti-IgG
15' PdBu

42kD —

DISCUSSION

Many receptors employ similar second-messenger reactions in the transduction of their signals. For example, the growth response of quiescent fibroblasts to serum-derived growth factors and the response of B cells to mIg occupancy share several second messenger pathways. Both systems activate protein tyrosine kinases, causing tyrosine phosphorylation of a similar set of protein substrates, such as PI-3 kinase, the GTPase activating protein of *ras*, MAP kinase, and PLCγ. Subsequent to and dependent on the receptor-activated tyrosine kinase activity is the hydrolysis of PIP_2, leading to the activation of PKC and Ca^{2+}-dependent events. The serum stimulation of fibroblasts causes the induction of more than 80 early response genes (27), and four of these have already been reported to be activated by mIg cross-linking in B cells (*c-myc*, *c-fos*, *egr-1*, and *jun-B*). We examined seven additional early response genes and found that mIg-derived signals activated four of these genes in resting splenic B cells and two or three of these four in two B lymphoma-derived cell lines. In each case, induction of these genes was not blocked by the protein synthesis inhibitor CHX, indicating that pre-existing proteins were capable of mediating the induction. The products of these additional early response genes induced in B cells include two putative transcription factors and two proteins of unknown function. The *nur77* product has homology to the DNA-binding and ligand-binding domains of steroid hormone receptors (58), whereas the *nup475* product is capable of binding Zn^{2+} and is found in the nucleus of fibroblasts (59). *pip92*, encodes a cysteine-rich, highly labile protein found in the cytoplasm of fibroblasts (60) and as such is the first non-nuclear early-response gene product reported to be

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induced by anti-Ig in normal B cells. The sequence of *3CH134* indicates that it encodes a protein phosphatase (61).

In addition to being induced by mIg in B cells and by growth factors in fibroblasts, several of these genes are induced in response to stimuli that elicit diverse responses in other cell types (62, 63). While some of the early response genes induced in these systems are common, there are also differences. For example, the gene *3CH134* is not expressed in the cell line WEHI-231, which undergoes growth arrest, while it is induced in the cell line 2PK-3, which represents a stage of development that has a positive growth response to mIg-derived signals. It may be that different biological outcomes caused by the various signals are realized at the level of the particular subset of early-response genes activated.

We also explored the mechanism by which mIg signal transduction induced mRNA expression of these early response genes. Stimulation of PIP_2 breakdown by means of an introduced HM1 acetylcholine receptor also resulted in an increase in the mRNA levels of early response genes in the transfected 2PK-3 cell line. Additionally, the induction of these four early response genes by phorbol esters occured in B cells, as it does in fibroblasts. The phorbol ester-inducibility of these genes in B cells suggests that PKC is at least partly responsible for mediating their "turn on". This conclusion is supported by the observation that the induction of all three of the early response genes tested was inhibited by the PKC inhibitor compound 3, regardless of whether the induction was triggered by anti-Ig, phorbol esters, ionomycin, or carbachol acting through the introduced muscarinic acetylcholine receptor. One gene, *3CH134*, appeared to be primarily induced

by elevated intracellular calcium levels in 2PK-3 cells (in contrast to splenic B cells). Even this induction was inhibited by compound 3, suggesting that a protein kinase, possibly PKC active at a low constitutive level, may play a role.

The mechanism by which PKC promotes the activation of *pip92, 3CH134,* and *nup475* is not evident. Phorbol esters have been reported to activate a number of transcription factors, including members of the AP-1 family (28), NF-kB (29), and serum response factor (SRF) (64). Transcriptional regulatory regions have not yet been identified for the four early response genes we have examined. Current evidence suggests that the AP-1 family of DNA-binding proteins, which trans-activate transcription through the TRE , may not play a part in inducing early response genes in B cells. Single TRE-driven reporter genes are not activated by anti-IgM in the B cell line Bal 17 (65). TRE-binding proteins do appear in B cells stimulated with anti-Ig, but with delayed kinetics, suggesting that they may affect secondary gene expression (65, 66). Moreover *jun-B*, which has a negative effect on the activity of single TRE sites under circumstances examined to date (67), is induced by anti-Ig in B cells (33). Interestingly, available data on promoter function do not indicate that a TRE is important for the activation of other early-response genes in response to serum in fibroblasts (68-70), with the possible exception of c-*jun* (71), which is not induced by mIg in B cells. NF-kB element-binding proteins are partially active in unstimulated B cells but are further activated after anti-Ig treatment of normal B cells (72, 73). The control of the early-response gene c-*myc* has been shown to be dependent on an NF-kB-like element in WEHI-231 cells (74). NF-kB elements have not been shown to be important for any of the other early response genes induced in B cells.

Finally, SRF is another potential trans-activator of early-response-gene induction in B cells. Several SRE are present in the regulatory region of the *egr-1* gene, and one of them has been shown to be important for *egr-1* induction in B cells (75). This element is also important for the induction of c-*fos* in fibroblasts, although this has not been tested in B cells yet. The mechanism by which serum or phorbol esters can activate through the SRE is not well established. One interesting possibility is that the activity of the SRE may depend on phosphorylation of the transcription factor p62^{TCF} by MAP kinase (76). In some circumstances, induction of binding to the SRE of the ternary factor p62^{TCF}, together with the constitutively bound SRF, correlates with induction of transcription through the SRE (76, 77). Gille et al showed that MAP kinase can phosphorylate p62^{TCF} *in* vitro, and that MAP kinase activation correlates with p62^{TCF} binding *in* vivo. Additionally, SRF can be phosphorylated by CKII (78, 79). CKII activity is increased in B cells by contact with activated T cells, as well as by treatment with phorbol esters and ionomycin (80), although the kinetics of its activation is too slow to account for the early gene induction observed here. It is not known whether CKII activity is also increased by signaling through mIg. In summary, NF-kB and SRF appear to be good candidates for mediating the PKC-dependent induction of early response genes caused by signaling through mIg, whereas AP-1 proteins appear to be less likely to do so.

We have found that four additional early response genes from fibroblasts are induced by mIg signaling in B cells. Phosphoinositide signaling and protein kinase C appear to play important roles in triggering these gene induction events. Clearly, our understanding of how mIg induces early-response gene

expression is still rudimentary. Greater understanding of this process will be important for understanding the role of individual second-messenger reactions in mediating the biological effects of antigen contact on B cells. In addition, identification of the early-response genes induced is also a first step toward developing a model for how changes in gene expression underlie the physiological events observed in B cells following Ag contact.

Abbreviations: mIg, membrane immunoglobulin; PIP$_2$, phosphotidylinositol 4,5-bisphosphate; HM1, human muscarinic receptor type 1; anti-Ig, anti-immunoglobulin antibodies; CHX, cycloheximide; PdBu, phorbol dibutyrate; GAPDH, glyceraldehyde-3-dehydrogenase; PKC, protein kinase C; PLC, phospholipase C; CKII, casein kinase II; SRF, serum-response factor; SRE, serum-response element; NF-kB, nuclear factor kappa B; AP-1, activating protein 1; TRE, TPA-response element.

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Chapter 2.

Induction of apoptosis of WEHI-231 B lymphoma cells by antigen receptor signaling and by Myc action

ABSTRACT

A subset of transformed B cell lines, including WEHI-231, exhibit apoptosis after stimulation of their antigen receptors. These cell lines serve as models for the clonal deletion of B cells expressing self-reactive antigen receptors. Because the product of the c-*myc* proto-oncogene has recently been implicated in the apoptotic process in fibroblasts and T lymphocytes, I have examined the role of this gene in the apoptosis of WEHI-231 cells. As previously reported, antigen receptor stimulation of these cells caused a rapid increase in c-Myc expression. To examine the effect of increased c-Myc expression on WEHI-231 cells, I introduced an estrogen-regulatable form of c-Myc (MycER) into these cells and found that activation of its product triggered the cells to undergo apoptosis. Moreover, the presence of the uninduced MycER fusion protein accelerated the onset of antigen receptor-induced apoptosis, presumably because of baseline activity of the fusion protein. Treatment with lipopolysaccharide, which substantially delays the onset of antigen receptor-mediated apoptosis in WEHI-231 cells, did not protect against MycER-mediated cell death. Conversely, stable overexpression of the proto-oncogene *bcl-2* delayed MycER-mediated but not antigen receptor-mediated apoptosis. Thus, although a role for c-*myc* in antigen receptor-mediated apoptosis of WEHI-231 is suggested by the synergy observed with the MycER protein, these observations indicate that antigen receptor signaling regulates another activity within the cell that promotes cell death, perhaps by cooperating with c-Myc function.

INTRODUCTION

The proper functioning of the immune system requires the activation of lymphocytes specific for foreign antigens and the inactivation of lymphocytes reactive with self components. Crosslinking of membrane immunoglobulin M (mIgM) with multivalent antigen or anti-IgM antibodies causes resting mature B cells to enter the G_1 phase of the cell cycle and increases their responsiveness to helper T cell-derived cytokines that promote proliferation and differentiation (1). In contrast, antigen receptor stimulation of immature B cells causes their disappearance or induces them to enter a nonresponsive state (2, 3). Mature B cells also can be inactivated or induced to die by antigen (4), particularly if they contact antigen without receiving T cell help. The former events promote production of antibodies in the immune response, whereas the latter events contribute to immunological tolerance to self components and downregulation of immune responses.

The death of autoreactive immature and mature B cells is thought to occur by apoptosis. Apoptosis is a cell suicide pathway by which deleterious or unnecessary cells are eliminated without the induction of an inflammatory response (5). It is characterized by the condensation of chromatin, the cleavage of DNA into fragments that are multiples of 200 base pairs, and the budding off of membrane-bound fragments of the cytoplasm. Apoptotic cells rapidly undergo phagocytosis—typically by macrophages. Apoptosis has been observed in antigen receptor-stimulated CD4 and CD8 double positive thymocytes (6-8) and in mature Ly-1 B cells located in the peritoneum (4), although direct *in vivo* evidence for the apoptosis of normal immature B cells has not yet been presented. A subset of B cell lines derived from

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leukemias and lymphomas undergo apoptosis upon treatment with anti-IgM antibodies used as a surrogate for antigen (9, 10). Such cell lines serve as models for the antigen receptor-induced inactivation of B lymphocytes. The most actively studied of these cell lines is WEHI-231 (11), which responds to antigen receptor stimulation by reducing its cell volume (12), arresting in G_1 phase of the cell cycle (13, 14), and initiating apoptosis (10, 15).

Recently the c-*myc* proto-oncogene has been found to play a role in the initiation of apoptosis. For example, when cells expressing a deregulated c-Myc protein introduced by transfection are rendered quiescent by lymphokine or serum withdrawal, conditions under which c-Myc expression is normally reduced (16-20), a large proportion of these cells undergo apoptosis (21, 22). In addition, the activation-induced apoptosis of several lymphoid cell lines can be blocked by treatment with c-*myc*-specific antisense oligonucleotides. This was shown for a T cell hybridoma stimulated through the T cell antigen receptor (23), a Burkitt's B lymphoma treated with a calcium ionophore (24), and antigen receptor-mediated apoptosis of WEHI-231 cells (25). There is some uncertainty regarding the interpretation of these experiments, however, because the antisense effect in B cell lines may not be due to a specific effect on c-Myc expression (26). In any case, the importance of c-*myc* for activation-induced apoptosis in T cell hybridomas has been further supported by experiments with dominant negative proteins designed to interfere with c-Myc (27). Thus, c-*myc* likely plays a role in apoptosis in a variety of situations. Because antigen receptor stimulation of WEHI-231 cells leads to a rapid increase in c-*myc* mRNA and c-Myc protein levels, it may be that c-*myc* mediates the apoptotic effect in these cells. We report here that overexpression of c-Myc can induce apoptosis in the WEHI-231 cell line. This

differs from the effects of anti-Ig treatment on these cells in a number of ways, however, suggesting that antigen receptor-induced apoptosis of WEHI-231 cells must be mediated by additional events that initiate apoptosis either alone or in conjunction with increased c-Myc.

MATERIALS AND METHODS

Cells and cell culture The mouse B lymphoma cell line WEHI-231 was cultured at 37°C in an atmosphere containing 5% CO_2 in RPMI 1640 medium (M.A. Bioproducts, Walkerville, MD) supplemented with 10% heat-inactivated fetal calf serum (Hyclone, Logan UT), 1 mM sodium pyruvate, 2 mM glutamine and 50 μM 2-mercaptoethanol (WEHI-231 medium). WEHI-*mycER* cells were cultured in the same medium lacking phenol red and containing 1.9 mg/ml G-418 (Gibco-BRL, Gaithersberg, MD). Cell lines were grown in logarithmic phase at cell densites of less than 5 x 10^5/ml. Gentamycin (50 μg/ml) was added to all experimental cultures.

Reagents Goat anti-IgM (Jackson Immunoresearch Laboratories, Westgrove, PA) antibodies were used at 1 μg/ml. β-estradiol (Sigma Chemical Co., St. Louis, MO) was added at 1 part per 100 from dilutions made in ethanol.

Introduction of genes encoding exogenous proteins into cell lines
Subconfluent ψ-2 cells were transfected with the pLNCX vector expressing *mycER* (28) and then supernatants were harvested after 24 hours of incubation in nonselective WEHI-231 medium at 37°C in an atmosphere containing 5% CO_2. These supernatants were added to cultures of WEHI-231 cells so that the viral supernatant represented 50% of the volume. Polybrene (Sigma) was added to 8 μg/ml and the cultures were incubated for 4 hr. Next, the virus-containing medium was washed out and replaced with WEHI-231 medium containing 8% fetal calf serum that had been treated with activated charcoal to remove steroids. The infected cells were grown for two more days, and the medium was replaced with the same medium supplemented

with 1.9 mg/ml G418. At this point cells were plated at appropriate densities in microtiter plates in 0.25 ml/well, and clones were obtained two weeks later. Charcoal treatment resulted in reduced viability, possibly due to removal of some other serum component, and subsequently the infected cells were cultured in media containing 5% complete fetal calf serum but in this case lacking phenol red.

The plasmid pLPC-*bcl-2* was created by transferring the human *bcl-2* gene into the unique EcoR1 site in the vector pLPCX, which was derived by replacing the *neo* gene of the vector pLNCX with a gene encoding puromycin resistance (P. Roth, UCSF, unpublished). Twenty μg of plasmid DNA, linearized with Sca-1, was electroporated using a "Gene Pulser" (Bio-Rad, Hercules, CA) into 4×10^6 WEHI-*mycER* cells that had been washed once and resuspended in 0.8 ml 20 mM HEPES, 137 mM NaCl, 5mM KCl, 0.7 mM Na_2HPO_4, and 6mM dextrose, pH7.2 in a 4 mm cuvette (Bio-Rad). A pulse of 500 μF @ 320 V was discharged into the culture after which the cuvette was placed on ice for 10 minutes. The cells were placed in 15 ml non-selective culture medium for 2 days, after which 10^4 viable cells in 1 ml culture medium supplemented with 1.0 mg/ml G418 and 0.5 μg/ml puromycin were placed in each well in 24 well plates. Individual colonies were collected after 10-12 days of growth in selective medium.

Cell cycle analysis Cell cycle analysis was performed as described (14). 10^6 cells were pulsed with 15 μM BrdU for 30 min, pelleted by centrifugation, fixed in ice-cold 70% ethanol, and treated for 20 min with 0.5% Triton X-100 in 2M HCl. After washing three times in PBS with 0.5% Tween-20 (PBS/Tw), the cells were resuspended in 50 μl PBS/Tw and 20 μl FITC-conjugated anti-BrdU

antibodies (Becton Dickenson, San Jose, CA), and incubated for one hour. The cells were then washed four times in PBS/Tw, resuspended in PBS/Tw containing 5 µg/ml propidium iodide, and analyzed on a flow microfluorimeter (FACSCAN, Becton Dickenson), using the LYSIS II program (Becton Dickenson). The BrdU vs propidium iodide plots presented in Figure 4 were derived from data that had been gated by forward and side scatter to exclude clumps of cells. This procedure had negligible effect on the cell cycle distributions.

RNA isolation and northern blot analysis Total RNA was extracted from cells by the acid phenol method (29) with the exception that only a single isopropanol precipitation was performed. RNA (2.5 µg/lane) was resolved by electrophoresis through a 1% agarose gel containing 2 M formaldehyde, buffered with 5 mM Hepes, pH 7.0, transferred to a "Genescreen" membrane (NEN Research Products, Boston, MA), and crosslinked for 1 min to the membrane by UV irradiation by a Stratalinker 1800 (Stratagene, La Jolla, CA). Specific RNA was detected by probing the membrane with a random oligonucleotide-primed (Boehringer Mannheim Biochemicals, Indianapolis, IN) ^{32}P-labeled cDNA probe. Hybridization was performed in buffer containing 50% formamide, 1 M NaCl, 1% SDS, 10% dextran sulfate, and 125 µg/ml denatured salmon sperm DNA. The filter was washed in 0.1 X SSC and 1% SDS at 65°C to remove nonspecific binding of the probe. Equal loading of RNA was verified by ethidium bromide staining of the gel prior to transfer.

Immunoblotting Total cellular proteins (2 x 10^{4} cells per lane) (30) were separated on 8% SDS-PAGE gels and transferred to nitrocellulose by the semi-

dry method (E & K Scientific Products, Saratoga, CA). Membranes were probed with rabbit antiserum against the C-terminus of murine c-Myc (31) and sheep anti-mouse Ig-HRP (Boehringer Mannheim), and signals were detected by the enhanced chemiluminescence method (ECL, Amersham). Equal loading of proteins was verified by Commassie blue staining of a parallel gel, as well as of the experimental gel after transfer of proteins. Molecular weight determinations of blotted proteins were made by comparison with prestained marker proteins (Bio-Rad). The MycER protein was detected with rabbit antisera against a glutathione S-transferase-ER fusion protein (a gift from Steve Robbins, UCSF), sheep anti-rabbit Ig-HRP, and ECL (data not shown). Transfected human Bcl-2 proteins were detected with the anti-human Bcl-2 monoclonal antibody clone 124 (Dako, Carpinteria, CA)(diluted 1:200), followed by biotinylated sheep anti-mouse antibody (Jackson Immunoresearch, diluted 1:1,000), streptavidin-HRP (diluted 1:10,000), and ECL.

Immunoprecipitations Immunoprecipitations of Max and MycER proteins were performed as described in Blackwood et. al. (32), using the rabbit antisera described above and the C-17 anti-Max antisera from Santa Cruz Biotechnology Incorporated (Santa Cruz, CA). Material derived from 2×10^6 WEHI-*mycER* cells per lane was subjected to electrophoresis and immunoblotting as described above.

Cell proliferation and viability assays Cells were cultured between 2.5 and 5 X 10^4 cells/ml in 0.25 ml volumes at 37°C in 96-well flat-bottomed microtiter plates (Costar, Cambridge MA). At the indicated times, samples were removed from resuspended cultures and the number of cells able to exclude

trypan blue was determined with a hemocytometer. At least 200 cells were counted. Experiments were performed in triplicate. For determination of cell viability, cells were also analyzed for reduction of MTT, which measures mitochondrial activity, as described by Mossman (33). Cells were cultured with reagents, which were added in 10 µl volumes at approriate dilutions, in 96-well plates in a total volume of 100 µl, for the indicated times. At the end of the experimental incubations, 10 µl of 5 mg/ml MTT (3-(4,5-dimethylthiazozl-2-yl)-2,5-diphenyl-tetrazolium bromide, Sigma) in phosphate-buffered saline, pH 7.5, was added, and cells were cultured at 37°C for an additional 2 hours, at which time 200 µl isopropanol containing 40 mM HCl was added. Optical density was measured was measured on a Dynatech plate reader (Chantilly, VA) at a wavelength of 630 nM (reference wavelength was 570 nM). Experiments were performed in triplicate.

Visualization of apoptotic cells Cells were attached to a glass slide by cytocentrifugation for 5 min at 900 RPM, fixed in 4% paraformaldehyde for 10 min, washed several times with PBS, and incubated in the DNA stain 4,6-diamidino-2-phenylindole (DAPI) (Sigma) at 50 µg/ml in PBS. Cells were washed several times with PBS, coverslips were attached with Immumount (Shandon, Pittsburg, PA) and chromatin was visualized on a fluorescence microscope.

DNA fragmentation assay Cytoplasmic nucleic acids were obtained as described (34). Briefly, 5×10^6 cells were washed once with PBS and lysed in 0.6 ml buffer containing 10 mM Tris, 10 mM EDTA and 0.2% Triton X-100 (pH 7.5). After 10 min on ice, lysates were centrifuged at 10,000 x g at 4°C. The supernatant was extracted once with phenol and once with phenol-

chloroform:isoamyl alcohol (24:24:1). To the upper phase NaCl was added to 300 mM and nucleic acids were precipitated with 2 vol of ethanol. Following a rinse with 70% ethanol, the pellet was air-dried and dissolved in 30 μl of 10 mM Tris with 1 mM EDTA (pH 7.5). RNase A was added to 0.6 μg/ml and samples were digested at $37°$ for 30 min. Samples were resolved by electrophoresis in a 2% agarose gel containing 40 mM Tris-acetate and 2 mM EDTA, and nucleic acids were visualized by ethidium bromide staining.

Transient expression assay Transient transfections of WEHI-*mycER* cells were performed by using the DEAE-dextran method (35). 5 x 10^6 cells were transfected with 10 μg of the expression vector pMyc3E1bLuc (36), in the presence of 0.2 mg/ml DEAE-dextran for 30 minutes at room temperature. After 24 hours, the cells from a single transfection were divided and either left untreated or stimulated for the indicated times prior to harvesting. Cell pellets were resuspended in 50 μl 100 mM potassium phosphate and 1 mM dithiothreitol pH 7.8, and subjected to three cycles of freezing in a dry ice-ethanol bath followed by thawing in a $37°C$ water bath. The lysates were centrifuged for two minutes at room temperature in a microcentrifuge, and 25 μl of the supernatant was assayed for luciferase activity using 100 μl luciferase assay reagent (Promega Corporation, Madison, WI) on a Monolight luminometer (Analytical Luminescence, Arlington Heights, IL).

RESULTS

Anti-IgM treatment of WEHI-231 cells regulates c-*myc* mRNA and protein levels It has been reported that anti-IgM crosslinking of WEHI-231 cells leads to transient increases in c-*myc* mRNA and protein levels (37, 38). To verify that our subline of WEHI-231 cells exhibited similar behavior, c-*myc* mRNA and protein levels at various times after stimulation were examined by Northern blotting and immunoblotting (Fig. 7). As has been reported (37, 38), after an initial rise during the first 1 to 2 hours of stimulation, the levels of c-*myc* mRNA and protein fall to well below control values after approximately 12 hours. This is approximately when the cells begin to arrest in G_1 phase of the cell cycle, and when the oligosomal DNA fragments characteristic of apoptosis begin to appear in the cytoplasm (10, 15). Thus, it is possible that either the early transient increase or the subsequent decrease in c-Myc levels may play a causative role in the initiation of the apoptotic process.

Overexpression of exogenous c-*myc* can induce apoptosis To examine the role of changes in c-Myc activity on antigen receptor-induced apoptosis, I used retroviral infection to introduce into WEHI-231 cells a construct coding for a conditional form of the human c-*myc* gene product. This chimeric protein (MycER) comprises c-Myc fused to the ligand-binding domain of the human estrogen receptor (ER) (28). The addition of ligands such as β-estradiol or hydroxytamoxifen allows the c-Myc moiety of the chimera to become active (39). For example, β-estradiol treatment of Rat-1 fibroblast cells expressing this chimera causes them to become transformed (28). Under serum-starved conditions, activated MycER induces some of these cells to
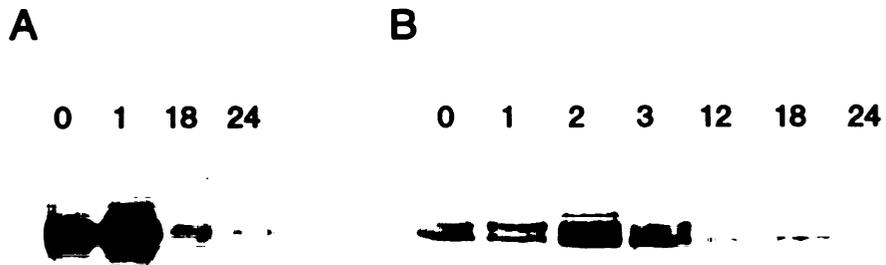
FIGURE 7. Induction of c-*myc* mRNA and protein expression following anti-IgM signaling in WEHI-231 cells. Cells were stimulated for the indicated times (in hours) with 1 μg/ml anti-IgM antibodies and total cell lysates were subjected to Northern (A) and Western (B) analysis.

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enter S phase of the cell cycle and subsequently divide (28), whereas other cells are induced to undergo apoptosis (21).

First, I examined the effect of c-Myc activity on the growth and survival of WEHI-231 cells by determining the number of viable cells recovered at various times after activation of the MycER protein with β-estradiol in WEHI-*mycER* cells. There was a marked effect of β-estradiol on cell recovery. Whereas the untreated cells grew rapidly (the doubling time is approximately 14 hours), β-estradiol caused a reduction in the number of viable cells by 24 hours and a greater reduction by 36 hours. Presumably activated MycER was inducing cell death despite the presence of normal growth medium. Interestingly, the rate of cell death after anti-IgM treatment alone was more rapid in the WEHI-*mycER* cells than it was in parental WEHI-231 cells. Whereas cell death was not complete in WEHI-231 cells before 48 hours, essentially no WEHI-*mycER* cells survived beyond 36 hours of anti-IgM treatment (Fig. 8 and data not shown). This acceleration was presumably due to baseline c-Myc activity of the uninduced MycER chimeric protein. Treatment of WEHI-*mycER* cells with the combination of β-estradiol and anti-IgM resulted in an even faster rate of death of the cells than did treatment with either stimulation alone. These results suggest that MycER action promoted cell death of WEHI-231 cells, both in combination with anti-IgM and by itself.

Several control experiments were performed to verify that the β-estradiol treatment and MycER chimera were exerting their effects as predicted. The effect of β-estradiol on cell death was apparently due solely to activation of MycER, inasmuch as β-estradiol had no effect on the rate of proliferation or

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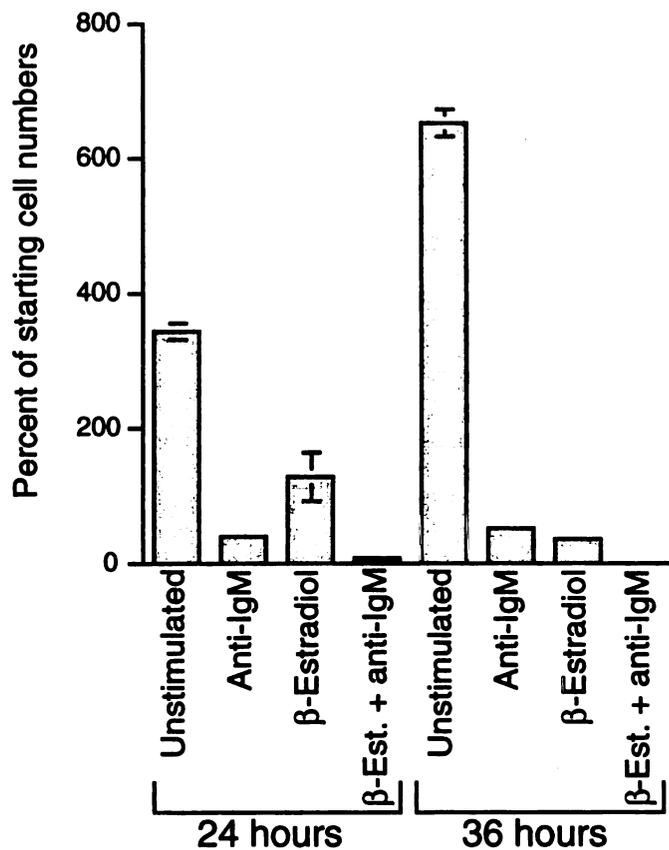
FIGURE 8. Effect of activated MycER protein on the growth and survival of WEHI-231 cells. WEHI-*mycER* cells were treated for the indicated times with 1 μM β estradiol, 1 μg/ml anti-IgM, or both. The number of viable cells recovered is expressed as a percentage of the number of cells present at the start of the culture. Error bars represent two times the standard error of the mean (S. E. M.). For many samples, the error bars were too small to be included.

on the rate of anti-IgM-induced cell death of the parental WEHI-231 cells (data not shown). Hydroxytamoxifen, an estrogen receptor antagonist that blocks transcriptional activity intrinsic to the portion of the estrogen receptor included in the chimera but nevertheless allows the MycER chimera to become activated (40), also inhibited cell growth and caused apoptosis in WEHI-*mycER* cells without affecting the growth of parental WEHI-231 cells (data not shown). The latter observations indicate that the c-Myc part of the MycER chimera, rather than the transcriptional activity derived from the ER domain, was responsible for inducing apoptosis in these cells.

β-Estradiol-treated WEHI-*mycER* cells appeared to die by apoptosis. The appearance of condensed chromatin (5), which is observable as intensely staining spots in DAPI-stained cytocentrifuged cell preparations, is characteristic of cells having undergone apoptosis. As shown in Fig. 9, the chromatin of dying cells resulting from either β-estradiol or anti-IgM treatment of WEHI-*mycER* cells was indistinguishable. Another feature of apoptosis is that chromosomal DNA becomes cleaved into small fragments that are multiples of ~200 nucleotides and appear as "ladders" on an agarose electrophoresis gel. Treatment of WEHI-*mycER* cells with either anti-IgM or β-estradiol resulted in the appearance of such DNA ladders (Fig. 10). Similar DNA ladders were seen with DNA from anti-IgM-treated WEHI-231 cells not expressing the *mycER* construct (data not shown). Thus, overexpression of c-Myc protein in WEHI-231 cells induced cell death by an apoptotic process similar to that induced by anti-IgM, at least by these two criteria.

MycER induces apoptosis in cells from different stages of the cell cycle in the absence of a cell cycle block The apoptosis induced by anti-IgM treatment of
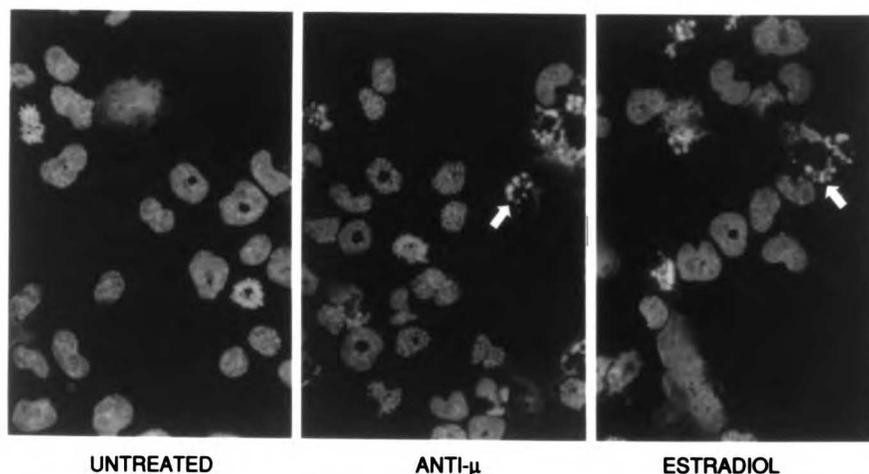
FIGURE 9. Anti-IgM- and MycER-mediated cell death of WEHI-*mycER* cells are both morphologically similar to apoptotic cell death. WEHI-*mycER* cells were treated for 12 hours with either anti-IgM or β-estradiol. The cells were then fixed, cytocentrifuged, and stained with the DNA stain DAPI. Some of the cells with condensed chromatin characteristic of apoptotic cells are indicated by the arrows.

FIGURE 10. Both anti-IgM and β-estradiol treatment of WEHI-*mycER* cells cause fragmentation of chromosomal DNA. WEHI-*mycER* cells were treated for 12 hours with either anti-IgM or β-estradiol. Cytoplasmic DNA was isolated and separated by electrphoresis on a 2% agarose gel and then stained with ethidium bromide. The lowest major bands represent DNA fragments 200 nucleotides in length.

WEHI-231 cells appears to occur in cells that have first arrested in the G_1 phase of the cell cycle (13-15). Therefore, I examined the effect of MycER activation on the cell cycle phase distribution of WEHI-*mycER* cells treated with anti-IgM or β-estradiol, as described above. Representative cell cycle data are shown in Fig. 11 and the results are summarized in Table 1. As can be seen in Table 1, with anti-IgM treatment, there was an increase over time in the number of cells in G_1 phase of the cell cycle, and this increase preceded the appearance of apoptotic cells having less than unit DNA content. In contrast, β-estradiol treatment of the cells did not lead to an increase in cells in G_1 phase but rather led to increased numbers of cells in the apoptotic region of the profile between G_1 and G_2/M phases (Apop* window in Fig. 11). The cells in this region—which appeared not to be replicating their DNA given that they did not incorporate BrdU—contained less than the amount of DNA required for G_2 phase and therefore may have been cells from S and G_2/M phases that had initiated apoptosis. The cells corresponding to this region of the profile were sorted from WEHI-*mycER* cells stimulated for 12 hours with β-estradiol, and the majority of these cells were seen to have condensed nuclei characteristic of apoptotic cells (data not shown). Thus, in contrast with apoptosis induced by anti-IgM, apoptosis induced by c-Myc was apparently not restricted to cells in G_1 phase of the cell cycle.

MycER protein binds Max protein and regulates gene expression The ability of c-Myc to mediate transformation (41) and apoptosis (42) appears to depend on formation of a heterodimer between c-Myc and the constitutively expressed Max protein. To determine whether the MycER protein also is capable of interacting with the endogenous Max protein, I immunoprecipitated Max protein from lysates of untreated and β-estradiol

FIGURE 11. Cell cycle analysis of cells undergoing apoptosis as a result of stimulation through either MycER or the antigen receptor. WEHI-*mycER* cells were treated for the indicated times with either anti-IgM or β-estradiol and then were pulsed with BrdU for 30 minutes, fixed, and analyzed by two color flow microfluorimetry for DNA synthesis (BrdU staining) and DNA content (propidium iodide staining). Windows used for determining the fraction of cells in each phase of the cell cycle are shown and identified in the first panel. "Apop" indicates apoptotic cells having less DNA than G_0/G_1 cells. "Apop*" indicates apoptotic cells with a higher DNA content/cell than the other apoptotic compartment.
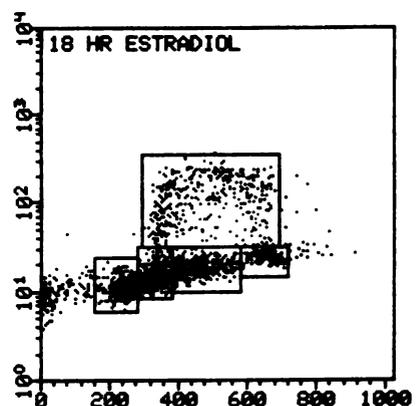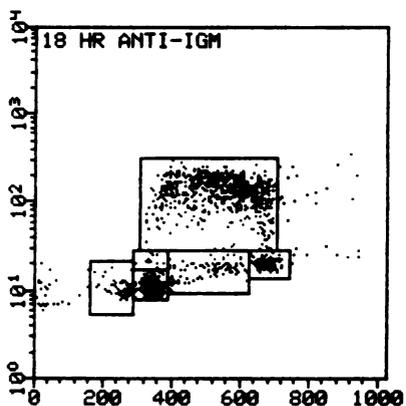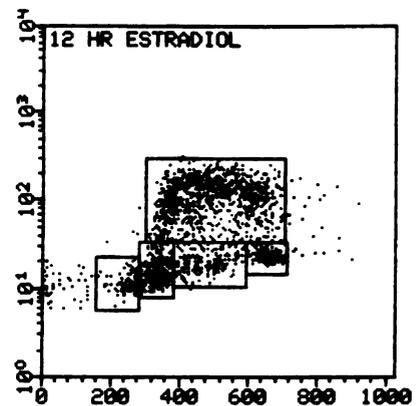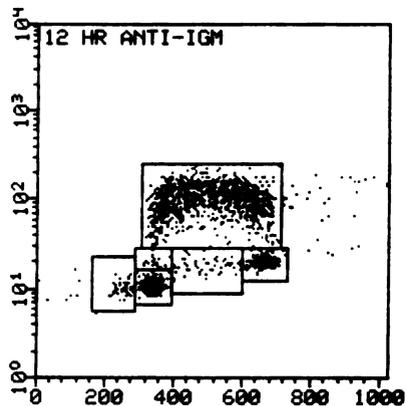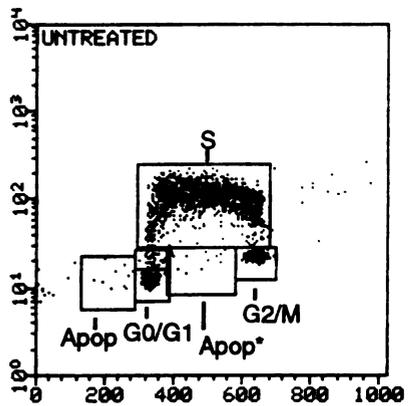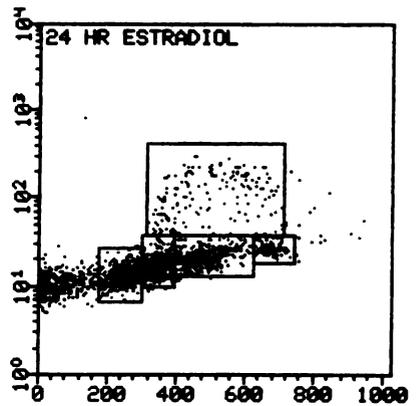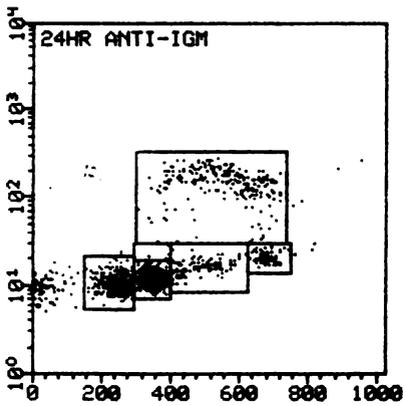
Propidium iodide fluorescence

BrdU incorporated

78

Treatment	Cell cycle stage (%)			
	G0/G1	S	G2/M	apoptotic
Unstimulated	14	68	14	1
12 hr β-estradiol	19	54	10	16
12 hr anti-IgM	28	58	9	5
18 hr β-estradiol	24	26	8	42
18 hr anti-IgM	48	35	8	9
24 hr β-estradiol	23	16	6	55
24 hr anti-IgM	54	19	5	23

TABLE 1. Cell cycle distributions of WEHI-*mycER* cells stimulated by cross-linking the antigen receptor or by hormone activation of the introduced MycER chimera. The boxed regions in the flow microfluorimetry plots of Fig. 4 are represented here as a percentage of total cells.

stimulated WEHI-*mycER* cells and examined coprecipitation of the MycER

protein by immunoblotting with an anti-ER antibody (Fig. 12). Surprisingly,

both the inactive form and the active form of MycER associated with Max to a

similar degree. Correspondingly, immunoprecipitation with an antibody that

recognized the ER part of the MycER protein (anti-ER) coprecipitated

equivalent amounts of Max with or without β-estradiol treatment. This

behavior differs from that of MycER in Rat-1 fibroblasts (43), where Max does

not associate with MycER until activated by β-estradiol. In either case, the ER

domain of the MycER chimera inhibits function until β-estradiol is added, at

which point its c-Myc-like function is revealed. Sequential

immunoprecipitations with anti-Max antibodies failed to fully deplete the

lysates of MycER, in both β-estradiol-treated and untreated cells, suggesting

that a fraction of the MycER proteins were not complexed with Max. Given

that additional Max was present in the cell, we postulate that the MycER

protein not bound to Max was material that had been irreversibly inactivated

by the ER domain.

The ability of MycER to form a complex with Max suggested that MycER

functions as a transcriptional activator, as seen with the normal c-Myc

protein. Overexpression of exogenous c-Myc has been shown to result in

downregulation of endogenous c-*myc* mRNA and protein in dividing

fibroblasts (44). In WEHI-*mycER* cells, β-estradiol treatment caused a dramatic

reduction in endogenous c-*myc* protein and mRNA within 1.5 hours (Fig. 13

and data not shown). This result is consistent with the MycER protein

possessing the negative transcriptional regulatory property that has been

ascribed directly or indirectly to the c-Myc protein. Beyond 1.5 hours, the

level of endogenous c-Myc increased, although the level was still lower than
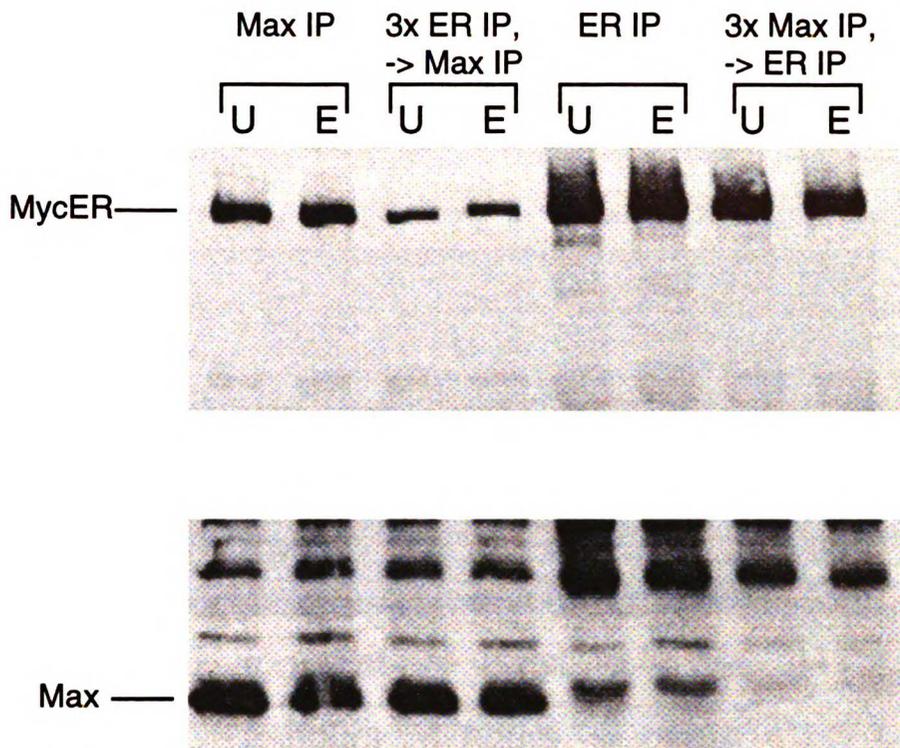
FIGURE 12. MycER protein associates with endogenous Max protein in WEHI-*mycER* cells. WEHI-*mycER* cells were treated with β-estradiol for two hours or left untreated. As indicated in the figure, lysates were either immunoprecipitated once with anti-Max or anti-ER or were immunoprecipitated three times sequentially with anti-Max or anti-ER antibody and then immunoprecipitated with the other antibody. After resolution by SDS-PAGE and transfer to nitrocellulose, the MycER and Max proteins were detected by immunoblotting. The filters were probed seperately with either anti-ER antibodies (upper half) or anti-Max antibodies (lower half).

U   α-μ   Est   α-μ   Est   α-μ   Est   U

2 hours   4 hours   8 hours
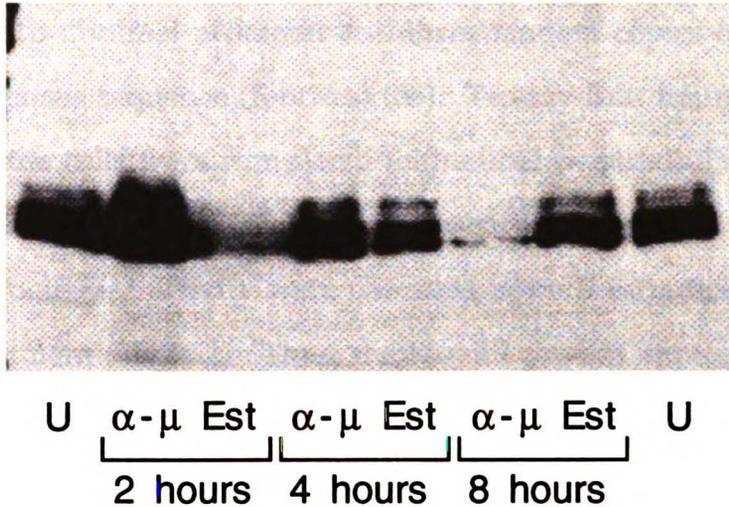
FIGURE 13. Activation of the MycER protein by addition of β-estradiol causes reduction in c-Myc protein in WEHI-*mycER* cells. Western blot analysis of the levels of c-Myc protein present in WEHI-*mycER* cells that were stimulated for the indicated times with either anti-IgM (α-μ, 1 μg/ml) or β-estradiol (Est, 1 μM).

that of uninduced cells. This phenomenon may have been due to relief from the autoregulatory effects when a lower overall level of c-Myc (endogenous c-Myc + MycER) was attained. Next, I tested whether the mycER protein could transactivate transcription by binding to the consensus c-Myc-binding DNA sequence. WEHI-*mycER* cells were transiently transfected with a c-Myc-responsive reporter plasmid expressing the *luciferase* gene driven by the adenovirus E1B minimal promoter and three tandem copies of the c-Myc-binding consensus sequence (E-boxes) (36). Twenty-four hours after transfection, the cells were stimulated with either β-estradiol or anti-IgM antibodies for an additional 3.5 and 7 hours each. Approximately two-fold increases in luciferase activity were observed upon β-estradiol treatment or anti-IgM treatment (Table 2). Thus, the MycER protein appeared to function as a c-Myc-like transcriptional regulator by three criteria: its ability to complex with Max protein, its ability to down-regulate endogenous c-Myc expression, and its ability to increase E-box-dependent transcription.

Differential inhibition of antigen receptor- and MycER-mediated apoptotic pathways by LPS treatment and Bcl-2 overexpression Lipopolysaccharide (LPS) treatment substantially delays the onset of apoptosis of WEHI-231 cells that have been treated with anti-IgM (45), and this protection was also evident in WEHI-*mycER* cells, measured by the metabolic substrate MTT (33). In contrast, LPS had only a very small effect on cell death caused by β-estradiol (Fig. 14). Although LPS has been reported to cause a transient early induction of c-*myc* mRNA and protein in normal resting B cells (16), LPS treatment did not cause any early changes in the levels of endogenous c-Myc protein in anti-IgM- or β-estradiol-treated or untreated WEHI-*mycER* or WEHI-231 cells (data not shown). This observation, coupled with the failure of LPS to prevent

Relative luciferase activity

Treatment	3.5 hr	7 hr.
Unstimulated	1.0	N. D.
β-Estradiol	1.7	2.6
Anti-IgM	2.1	2.1

TABLE 2. Transcription of a reporter construct driven by a c-Myc-responsive promoter after anti-IgM stimulation or MycER activation. Twenty four hours after transfection with a reporter plasmid expressing a c-Myc-regulated *luciferase* gene, WEHI-*mycER* cells were stimulated for the indicated times with either 1 μM β-estradiol or 1 μg/ml anti-IgM antibodies. Luciferase counts are expressed relative to that seen with unstimulated cells.

MycER-induced apoptosis, suggests that the ability of LPS to block anti-IgM-induced apoptosis is not due to its interference with the induction or action of c-Myc.

Overexpression of the *bcl-2* proto-oncogene has been demonstrated to inhibit apoptosis in a number of circumstances, including some in which c-Myc is responsible (46, 47). In WEHI-231 cells, however, overexpression of *bcl-2* does not inhibit anti-IgM-induced apoptosis (48, 49). To determine whether MycER-mediated apoptosis is susceptible to modulation by Bcl-2, I introduced human Bcl-2 into WEHI-*mycER* cells by transfection. The Bcl-2 levels of 30 clones were measured by anti-Bcl-2 immunoblotting, and the susceptibility of the cells to growth inhibition by anti-IgM or β-estradiol or both was determined by the MTT assay. The clones were divided into three groups, based on the levels of exogenous Bcl-2 expression (data not shown). The group expressing the highest levels of Bcl-2 was protected significantly from killing by β-estradiol, whereas the group expressing the lowest levels of Bcl-2 was not (Fig. 15). In contrast, high levels of Bcl-2 did not significantly inhibit anti-IgM-mediated killing. Thus, Bcl-2 inhibited MycER- but not anti-IgM-mediated killing, whereas LPS interfered with the latter but not the former. These results argue against the simple hypothesis that c-Myc is solely responsible for mediating the apoptotic effect of anti-IgM.
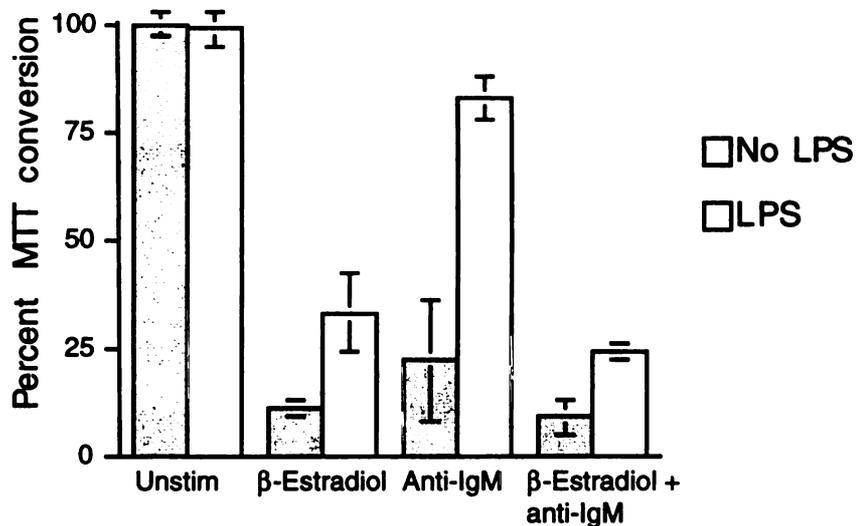
FIGURE 14. Effect of LPS treatment on death of WEHI-*mycER* cells induced by β-estradiol, anti-IgM, or the combination of these agents. WEHI-*mycER*cells were stimulated for 27.5 hours with the indicated treatments, pulsed with MTT for 2 hours, harvested, and the OD readings of the cultures were taken. Results are expressed as a percentage of the OD readings of untreated cultures at the time of harvest. Error bars represent two times the S. E. M.
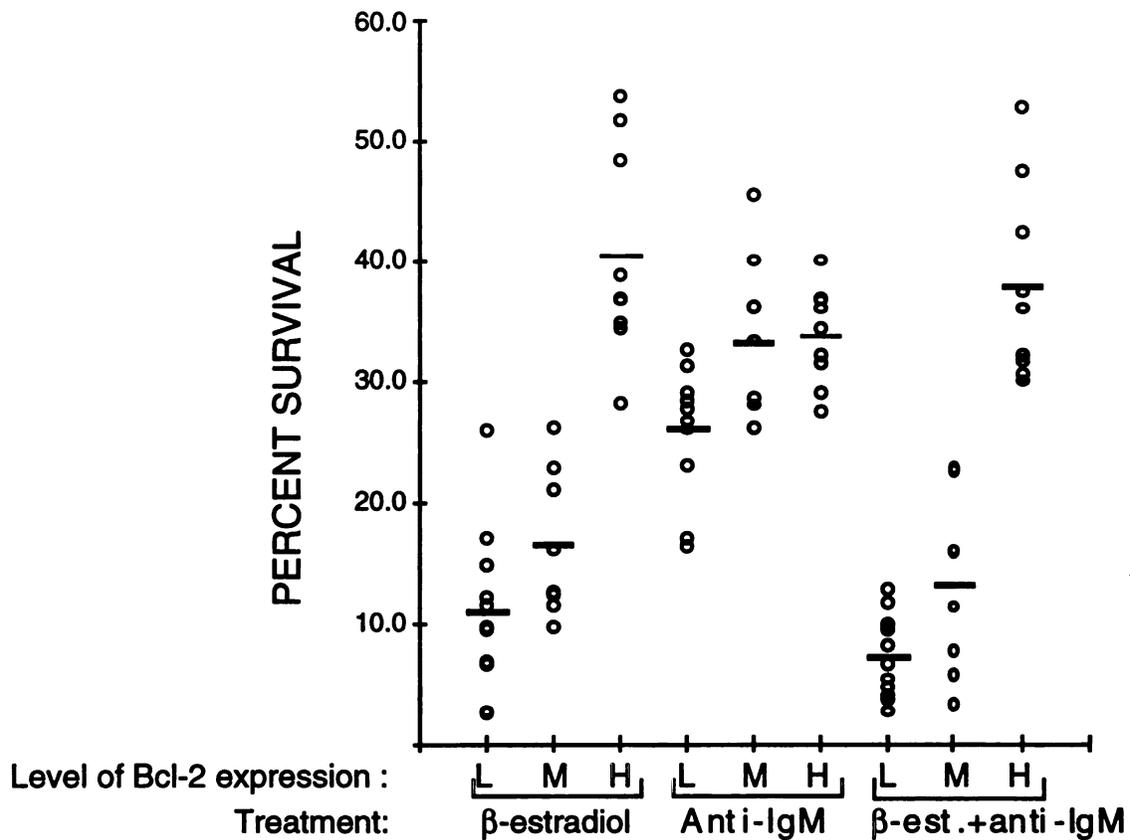
FIGURE 15. Effect of exogenous Bcl-2 levels on growth of β-estradiol, anti-IgM, or the combination of these agents. Scatter plot showing percent survival of the indicated treatments of groups of WEHI-*mycER* cell transfectants expressing low (L), medium (M), or high (H) levels of exogenous Bcl-2. WEHI-*mycER*cells were stimulated for 36 hours with the indicated reagents, labeled with MTT for 2 hours, harvested, and the ODs of the cultures were measured. The average values for each group are plotted as a solid horizontal line. Values for parental WEHI-*mycER* cells were: β-estradiol, 15%; anti-IgM, 32%; β-estradiol + anti-IgM, 21%.

DISCUSSION

Apoptosis has increasingly come into view as an important feature of immune regulation, but the intracellular events that determine whether a stimulated cell will be activated or will undergo apoptosis are poorly understood. Recent experiments suggest a role for the c-*myc* proto-oncogene product in the apoptosis of growth factor-starved fibroblasts and myeloid cells and of antigen receptor-stimulated lymphocytes (21-23). Crosslinking the antigen receptor leads to expression of c-*myc* as an immediate early gene induction in mature splenic B cells (50) and, as previously reported (37, 38) and confirmed here, in the immature B cell line WEHI-231. Therefore, I examined the possible role of c-*myc* in the antigen receptor-induced apoptosis of WEHI-231 cells. In our initial approach, I asked whether creating an increase in c-Myc would by itself be sufficient to bring about the apoptotic events seen after antigen receptor crosslinking. We used a regulatable form of c-Myc (the MycER chimera) to obtain inducible c-Myc function. Such upregulation of c-Myc function caused the WEHI-231 cells to rapidly undergo apoptosis. In addition, the presence of the uninduced MycER chimera in WEHI-231 cells resulted in an increase in the rate of onset of apoptosis after anti-IgM treatment, compared with untransfected parental cells--presumably a sign of some c-Myc activity in the absence of induction. The addition of β-estradiol to activate MycER caused a further increase in the rate of anti-IgM-induced apoptosis. These observations are consistent with there being a role for c-*myc* in anti-IgM-induced apoptosis, provided c-Myc is acting at a step that is limiting for the rate of apoptosis.

A number of control experiments were performed to examine the hypothesis that the chimeric MycER protein was in fact functioning like c-Myc. Coimmunoprecipitation experiments demonstrated that a significant fraction of the MycER in the cell was associated with Max, the normal cellular heterodimeric partner for c-Myc. Surprisingly, the amount of MycER/Max complex was not affected by β-estradiol treatment. Presumably β-estradiol acted upon MycER in the complexed form to activate the preformed dimers. In any case, the presence of MycER/Max dimers is consistent with MycER exhibiting c-Myc-like transcriptional regulatory function. This possibility was further tested in two ways. First, activation of the MycER fusion protein caused a decrease in the levels of endogenous c-*myc* mRNA and protein. The ability of c-Myc to down-regulate its own expression had been previously inferred from studies in which cells stably transfected with c-*myc* exhibited an inverse correlation between the levels of expression of introduced c-Myc and the levels of endogenous c-Myc (44). Second, activation of the MycER protein increased expression of a reporter construct driven by three repeats of the consensus Myc-Max heterodimer recognition sequence CAGCTG (36). Thus, MycER clearly exhibited c-Myc-like ability to regulate transcription. There is a theoretical possibility that MycER is a better transcriptional activator than c-Myc, because the estrogen receptor has transcriptional activating ability that could, in part, be contained in the region of the ER fused to Myc (40). However, the estrogen receptor antagonist hydroxytamoxifen blocks estrogen receptor transcriptional activating ability, but in our study hydroxytamoxifen acted as well as β-estradiol to induce apoptosis in WEHI-*mycER* cells. Thus, by these criteria, MycER was acting primarily as a regulatable form of c-Myc.

The sensitivity of WEHI-231 cells to apoptosis caused by expression of elevated c-Myc may reflect a similar sensitivity of normal immature B cells. In mice in which a transgenic c-*myc* gene was expressed in all B lineage cells, the pre-B cell compartment was expanded, whereas the B cell compartment was reduced (51). Larger numbers than normal of both types of B lineage cells were actively passing through the cell cycle (51). Thus, overexpression of c-Myc in these immature B cells appears to have caused increased proliferation. The decreased number of mature B cells suggests that elevated c-Myc levels were also accompanied by increased cell death, possibly because c-*myc* was promoting apoptosis under some circumstances. One possibility suggested by our experiments is that increased apoptosis was triggered by antigen receptor engagement of the Myc-transgenic immature or mature B cells.

Fibroblasts expressing MycER chimeric protein can be induced to undergo apoptosis by the addition of estrogen (21). However, in these fibroblasts, apoptosis occurred only under circumstances that did not support cell proliferation, such as when cells were placed in medium with a reduced amount of serum or treated with agents that block the cell cycle. These observations led to the hypothesis that apoptosis in fibroblasts is the result of significant expression of c-Myc at a time when cell cycle progression is blocked (21). The behavior of antigen receptor-triggered WEHI-231 cells may conform to this model, in that the antigen receptor-derived signal imposes a cell cycle block in G_1 phase, which precedes apoptosis (52). Moreover, attempts to obtain synchronized populations of WEHI-231 cells by interfering with the cell cycle through the use of cell cycle-inhibiting drugs or the withdrawal of serum lead to cell death, presumably by apoptosis (53). WEHI-231 cells constitutively express c-Myc at a moderate level, so this protein could be

important for apoptosis induction in these ways. In contrast with the observations in fibroblasts, however, apoptosis was induced in WEHI-231 cells by the activated MycER protein without an evident cell cycle arrest. This result suggests that WEHI-231 cells may differ from fibroblasts by being already "primed" for apoptosis (54), and therefore not requiring cell cycle inhibition to initiate this program for cell death.

The hypothesis that WEHI-231 cells are primed for apoptosis is supported by the observation that inhibitors of protein and RNA synthesis also induce apoptosis in WEHI-231 cells (48). Presumably, these cells already possess the components necessary for the apoptotic process; negative regulators needed to prevent apoptosis may be turning over rapidly and in this way become depleted upon inhibition of protein synthesis. C-Myc also is a rapidly turning over protein that would disappear in the course of treatment with protein synthesis inhibitors. C-Myc may, however, initiate processes that can proceed in the subsequent absence of negative regulators. Because c-Myc protein also would be absent during this time, its role might therefore be described as an early "switch" that triggers apoptosis unless counteracted in some way.

Similarly, several nontransformed lymphocyte populations may be primed for apoptosis. For example, immature CD4$^+$ CD8$^+$ thymocytes undergo apoptosis after antigen receptor signaling, treatment with glucocorticoids, or γ-irradiation (6). However, unlike their effect in WEHI-231 cells, protein synthesis inhibitors inhibit Ca^{2+}-, γ-irradiation- and glucocorticoid-induced apoptosis of thymocytes, and inhibit antigen receptor-induced apoptosis of T cell hybridomas as well (6, 55). Among mature lymphocytes, germinal center B cells proliferate rapidly while their immunoglobulin genes undergo

somatic mutation. Survival of those cells that express receptors with improved affinity for antigen is achieved by the receipt of an antigen receptor or CD40 signal to block the apoptosis that would otherwise occur (56). Thus, germinal center cells are primed for apoptosis, and antigen receptor signaling promotes cell survival. In contrast, in some other B cell populations, antigen contact induces apoptosis, as graphically illustrated in a line of transgenic mice expressing an immunoglobulin specific for an epitope on murine erythrocytes (4). These mice have very few B cells except in the peritoneum. These peritoneal B cells rapidly undergo cell death by apoptosis upon injection of erythrocytes into the peritoneum (4). Apoptosis is clearly a prevalent phenomenon among lymphocytes, and the primed state of WEHI-231 cells may be typical of many lymphocyte populations *in vivo*.

C-*myc* could play a role in the anti-IgM-induced apoptosis of WEHI-231 cells if the decision to enter apoptosis occurs quite early--for example during the initial transient rise in c-Myc levels. Whereas anti-IgM treatment of WEHI-231 cells leads to growth arrest and subsequent apoptosis, MycER induction led to apoptosis without growth arrest. This difference could result from the fact that there is a precipitous decline in c-Myc levels approximately 12 hours after anti-IgM stimulation. Because c-Myc activity is probably required for cell cycle progression (17, 18), this drop in c-Myc levels could cause growth arrest. According to this hypothesis, the failure of β-estradiol-treated WEHI-*mycER* cells to arrest in G_1 phase before entering apoptosis may simply indicate that c-Myc activity cannot decline in this situation. Nonetheless, apoptosis induced by c-Myc overexpression differed from that induced by anti-IgM in two other ways. LPS treatment prevented anti-IgM-induced apoptosis but had little effect on c-Myc-induced apoptosis. Conversely, expression of the

product of the anti-apoptotic gene *bcl-2* decreased c-Myc-induced apoptosis but not anti-IgM-induced apoptosis. These results argue against the simple model that antigen receptor-induced apoptosis is mediated solely by elevating c-Myc expression.

The ability of MycER induction to trigger apoptosis of WEHI-231 cells suggests that the antigen receptor could induce apoptosis by causing an elevation of endogenous c-Myc. Indeed, anti-IgM treatment does cause a rapid and transient rise in c-Myc levels. Several observations indicated that antigen receptor-induced apoptosis does not simply imply an elevation of c-Myc, however, as discussed above. Two more complicated models arise for the mechanism of antigen receptor-induced apoptosis of WEHI-231 cells. The first possibility is that *c-myc* does not participate in antigen receptor-induced apoptosis. Fischer et. al. have found that an antisense oligonucleotide specific for the translational start of *c-myc* can prevent anti-IgM-induced apoptosis of WEHI-231 and CH31 B cells (25). This result favors the possibility that *c-myc* plays an essential role in antigen receptor-induced apoptosis in these cells. However, it has recently been found that certain unmethylated CG-containing DNA sequences have activating properties for B cells (26), and the *c-myc* antisense oligonucleotide appears to contain such sequences. Therefore, additional experimental approaches will be required to assess whether or not *c-myc* participates in antigen receptor-induced apoptosis. The second possibility is that antigen receptor-stimulated *c-myc* participates in antigen receptor-induced apoptosis but that the antigen receptor stimulates at least one other intracellular event leading to apoptosis. Such a second pathway could act on the apoptotic machinery independently of *c-myc* or it could collaborate with *c-myc* in a number of possible ways. For example, the

activity of c-Myc could be modulated by phosphorylation by MAP kinase (57, 58), which is activated by antigen receptor signaling (59, 60) or by a related protein kinase (61). Alternatively, the second pathway could activate another transcription factor(s) that could cooperate with or modify the transcriptional activity or specificity of c-Myc. Given that Bcl-2 overexpression protects WEHI-231 cells from c-Myc-induced apoptosis but not antigen receptor-induced apoptosis, the second pathway could affect some component of the Bcl-2-related family of proteins and thereby neutralize the ability of endogenous Bcl-2 to protect against c-Myc-induced apoptosis. If, as in fibroblasts, c-*myc* drives cells through the cell cycle in the presence of a "survival signal" but induces apoptosis if expressed in the absence of the survival signal (62), another possibility is that the second pathway interferes with this survival signal (52). This final hypothesis, however, does not readily explain how the addition β-estradiol to WEHI-*mycER* cells induces apoptosis by itself.

The experiments presented here have demonstrated that c-Myc has the potential to induce apoptosis in WEHI-231 B lymphoma cells. The properties of this apoptosis, however, differed somewhat from antigen receptor crosslinking-induced apoptosis. We favor the hypothesis that antigen receptor signaling induces apoptosis by causing a transient elevation of c-*myc* expression and by generating an additional intracellular event that also participates in apoptosis. Additional experiments will be required to reveal the nature of this additional event and to test whether or not c-Myc indeed participates in antigen receptor-induced apoptosis.

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CONCLUSIONS

In this investigation I explored the potential role of early response gene inductions in mediating the action of the antigen receptor in B cells. The first step taken in these studies was to identify the major early response gene inductions that occur upon antigen receptor signaling in B cells. Although no early response genes uniquely induced in B cells were identified, I did find that a subset of a group of early response genes that are induced to high levels of expression in response to serum stimulation in fibroblasts (1) are also induced by mIg signaling in B cells. Two of these genes encoded transcription factors (2, 3), suggesting that they could be intermediaries in the genetic response to antigen receptor signaling. Another early response gene of B cells encoded a protein phosphatase that may inhibit the MAP kinase cascade (4). Thus, the genetic response to antigen receptor signaling employs elements in common with those of other receptors that regulate cell growth. Moreover, a number of the antigen receptor-induced early response genes are likely to play important biological roles in inducing subsequent genes and in regulating the signaling reactions.

Another goal of these studies was to understand the signaling pathways that connect the antigen receptor to the gene inductions. I found that activating the phosphoinositide signaling pathway, by means of an introduced muscarinic acetylcholine receptor expressed in the B lymphoma-derived cell line 2PK-3, was sufficient to induce expression of all three of the genes tested. Because PKC activation and increased intracellular calcium are the two principal "second messenger" events that result from phosphatidylinositol bis-phosphate hydrolysis (5, 6), I tested whether the genes could be induced by

mimicking each of those second messengers through the use of pharmacological agents. With one exception, in three different types of B cells, each of the four early response genes could be activated by treatment with phorbol esters, which activate PKC, and could not be activated by treatment with ionomycin, which elevates intracellular calcium levels. The exception was the gene *3CH143*, which was induced by ionomycin and not by phorbol esters in the 2PK-3 cell line, although it behaved like the other genes in normal splenic B cells. Use of the PKC inhibitor compound 3 demonstrated that all of the gene inductions, whether by cross-linking of the antigen receptor or by addition of the second messenger mimicking agents, were dependent on PKC. These results leave open the question of the function of the other signaling events triggered by antigen receptor engagement in B cells, because the phosphoinositide pathway appeared to be primarily responsible for the gene inductions examined. A complementary approach would be to test these genes for induction through receptors that induce these other signaling events, but without inducing the phosphoinositide pathway. A receptor that may meet these conditions is the CSF-1 receptor (7), which could be introduced into the B cell lines by transfection.

The second part of this investigation dealt with studying the biological role of one particular early response gene, c-*myc*, in antigen receptor-stimulated B cells. The c-*myc* gene was chosen because the recent literature had implicated it in the antigen receptor-mediated apoptosis of a T cell hybridoma and because of the ability to manipulate c-Myc activity in the cell (8). Unfortunately, the antisense oligonucleotide initially used to demonstrate a need for c-*myc* in apoptosis (9) was found to stimulate a protective signaling

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pathway unrelated to c-*myc* in B cells (10). Taking the opposite approach, I asked what the effect of overexpression of c-Myc would be on the mIg-mediated apoptosis of WEHI-231 cells. I found that activating a hormone-regulated chimeric Myc protein (MycER) activated apoptosis on its own and synergized with the mIg signal to induce more rapid apoptosis. One interpretation of the latter result is that expression of MycER was simply increasing a process that is rate-limiting for apoptosis—namely, the c-Myc induction. According to this interpretation, c-*myc* would play a role in triggering apoptosis through antigen receptor-stimulation.

Other observations, however, lead to the conclusion that the induction of c-Myc in response to mIg cross-linking was insufficient to mediate apoptosis by itself. The elevation of Myc activity achieved with the MycER chimera was sufficient to induce apoptosis, but this apoptosis was not preceded by the growth arrest that precedes antigen receptor-induced apoptosis. This difference could be indicative of the different dynamics of Myc activation in the two circumstances. The growth arrest seen with antigen receptor cross-linking could be attributed to a requirement for Myc to drive cell cycle progression coupled with the falling c-Myc levels seen 12 hours after anti-IgM treatment. When the MycER chimera was activated, however, activity of Myc was likely to be continuous, which would be compatible with continued cell cycling. There were, nevertheless, several other differences between antigen receptor-induced apoptosis and MycER-induced apoptosis. First, LPS, which delays the onset of antigen receptor-induced apoptosis (11), did not affect MycER-induced apoptosis. Conversely, overexpression of Bcl-2 (12), which does not protect against antigen receptor-induced apoptosis of WEHI-231 cells (13), did protect against Myc-induced apoptosis of these cells. The

observations with *bcl-2* appear to be inconsistent with a sufficient role for c-*myc* in antigen receptor-mediated apoptosis. One possibility is that antigen receptor signaling increases c-*myc* expression and acts to interfere with Bcl-2 in some way. The differential effects of LPS on antigen receptor-induced and MycER-induced apoptosis suggests that antigen receptor-induced apoptosis is mediated by events other than c-*myc* elevation, because LPS does not block c-*myc* elevation. It is possible, however, that the antigen receptor induces another event that acts in conjunction with elevated c-*myc* to induce apoptosis and that LPS interferes with this additional event. According to this hypothesis, MycER activation would have to lead to higher or more prolonged c-*myc* action than is seen in response to anti-IgM in order to explain how it can induce apoptosis without this second event.

To resolve the uncertainties regarding the existence of and the relation between possible multiple pathways leading from the antigen receptor to apoptosis, it is obviously imperative to obtain direct evidence for or against participation by c-Myc protein in the antigen receptor-mediated apoptosis of WEHI-231 cells. Attempts to implicate or absolve c-*myc* by stably transfecting WEHI-231 cells with proven dominant negative alleles (14, 15) of c-*myc* have been unsuccessful. This is probably because c-*myc* is needed for growth of the WEHI-231 cells and therefore inhibition of Myc function is not compatible with cell growth. A role for c-*myc* in the antigen receptor-induced apoptosis of a T cell hybridoma has been demonstrated through the transient expression of dominant negative c-*myc* and of native *max* (16). This T hybridoma has the ability to express transiently transfected genes in a majority of cells, and thus evaluations of cell survival could be made on the bulk populations of cells. Transiently transfected genes are expressed only in

a tiny fraction (<0.5%) of WEHI-231 cells, so a similar assay will necessitate having a means of identifying and assessing the viability of those cells that take up DNA, without perturbing that assessment. Such an assay has been used successfully to implicate the *nur77* gene in the antigen receptor-mediated apoptosis of a T cell hybridoma (17). The authors marked those cells that expressed a dominant negative allele of *nur77* with the co-transfected CD20 molecule, which was functionless in the cells.

As mentioned above, it is likely that a non-Myc-mediated event plays a part in antigen receptor-induced apoptosis of WEHI-231 cells. It is possible that the lack of *3CH134*, one of the early response genes induced in normal B cells, and not expressed in WEHI-231 cells could be responsible for the apoptotic outcome to mIg signaling. Its product, MKP-1, is a protein phosphatase and has been shown to specifically dephosphorylate and inactivate MAP kinase *in vitro* (4) and to block MAP kinase-mediated mitogenesis *in vivo* (18). MAP kinase has been shown to be activated by mIg signaling in WEHI-231 cells (19, 20), and the Ras pathway has been implicated in the induction of one of the early response genes, *egr-1* (21, 22), suggesting that MAP kinase is functional in these cells. The lack of MKP-1 expression in WEHI-231 cells may lead to prolonged MAP kinase activation, which could play a role in triggering apoptosis. This prolonged activation is of interest because of reports that c-Myc is a potential substrate for MAP kinase (23). Thus, prolonged phosphorylation of c-Myc by MAP kinase could be a hypothesized second event. This modification of c-Myc could potentiate its normal function, which would be consistent with the ability of MycER to induce apoptosis by itself. One prediction of this hypothesis is that overexpression of an active or

activatable allele encoding MAP kinase would prevent LPS from interfering with antigen receptor-induced apoptosis.

Another possibility is that the event that collaborates with c-*myc* in antigen receptor-induced apoptosis is the activation of another transcription factor. To test this hypothesis, one could use inhibitory strategies to examine whether this apoptosis was affected by blocking other transcription factors that are expressed in B cells, such as Egr-1, JunB, Nup475 and p53, or the c-Myc-interacting proteins such as Max and Mad. A more long range approach to this problem would be to isolate genes induced by activation of MycER and test these genes for sufficiency and necessity for the induction of apoptosis by the antigen receptor. Those genes that are found to be required for antigen receptor-mediated apoptosis may have regulatory regions that are controlled by factors that collaborate with c-Myc. For example, a gene whose overexpression has been shown to induce apoptosis in fibroblasts is *E2-F*, which encodes a transcription factor whose target genes are expressed in association with entry into S phase (24). Clarification of the possible epistatic relation of *E2F* to c-*myc* (25) may be a source of insight into how c-Myc interacts with the cell cycle machinery to regulate cell growth and apoptosis.

Finally, a variety of mutants of WEHI-231 have been made that do not arrest their growth or undergo apoptosis in response to mIg stimulation (26, 27). In one of these mutants, mIg stimulation leads to a prolonged elevation of c-*myc* mRNA levels. It was found that a 72 kDa protein that is tyrosine phosphorylated in response to mIg stimulation was missing in these mutant cells (26). This protein, which was identified as HS-1, could cooperate with c-*myc* to mediate the apoptotic response. The function of HS-1 is not known

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but its sequence suggests that it could be a transcription factor (T. Watanabe, personal communication). If c-Myc function is somehow inhibited in the mutant cells, then the elevated levels of c-*myc* mRNA could indicate inactivation of c-Myc's autoregulatory function. To test this hypothesis, it would be necessary first to reintroduce the HS-1 into the mutant cells to test for reconstitution of the growth arrest and apoptotic pathways. One would then ask whether the shutdown of c-*myc* expression was restored and, if so, look for other evidence of interaction of HS-1 with c-Myc, perhaps through physical association.

Studies of antigen receptor-induced apoptosis may have medical implications. For example, learning the details of c-*myc*'s role in apoptosis may be useful toward understanding the growth of tumors, especially those of B cell origin, such as Burkitt's lymphoma. Indeed, the behavior of c-Myc in WEHI-231 cells may be similar in Burkitt's B lymphoma cell lines (28) and in EBV-transformed B cells (29, 30). In addition, knowing details of the mechanism of tolerance induction in B cells may help in understanding how self-reactive B cells arise in the course of autoimmune diseases and could lead to the development of strategies for therapeutic intervention.

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