Cross-linking of Surface IgM in the Burkitt’s Lymphoma Cell Line ST486 Provides Protection against Arsenite- and Stress-induced Apoptosis That Is Mediated by ERK and Phosphoinositide 3-Kinase Signaling Pathways*

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The ST486 cell line, derived from a human Burkitt's lymphoma, is a model for antigen-induced clonal deletion in germinal center B-lymphocytes, with apoptosis induced upon cross-linking of surface IgM. Moreover, this cell line is highly sensitive to the induction of apoptosis by many chemicals, including sodium arsenite, a significant environmental contaminant with immunotoxic activity. In contrast to arsenite and other chemicals, surface IgM cross-linking induces apoptosis in ST486 cells with delayed kinetics. Moreover, the initial signaling events following IgM stimulation are associated with cell survival and proliferation and include activation of the extracellular-signal regulated kinase (ERK) and the phosphoinositide 3-kinase (PI3K) pathways. We examined the question of whether IgM-mediated activation of the ERK and PI3K pathways can influence the apoptotic response of ST486 cells following exposure to arsenite and selected drugs with different molecular targets, including cycloheximide, etoposide, and camptothecin, and a physical stress, hyperthermia. Our findings show that IgM-stimulated cells are significantly protected against arsenite and drug-induced apoptosis during a window of several hours after surface IgM cross-linking, as evidenced by an inhibition of cleavage of poly(ADP-ribose) polymerase and lack of morphological changes indicative of apoptosis. Significantly, surface IgM cross-linking also protects against arsenite-induced mitochondrial depolarization as well as caspase-9 cleavage. Furthermore, we demonstrate that this IgM-mediated protection requires the activation of the ERK and PI3K pathways, because inhibition of either pathway blocks the ability of antigen receptor activation to protect against apoptosis. Our study also provides evidence for p90S6 ribosomal kinase as a point of convergence between the two signaling pathways resulting in the phosphorylation of the pro-apoptotic Bcl-2 family member Bad at serine 112. This investigation demonstrates, for the first time, that specific signals transduced by activation of the B-cell receptor protect cells at a common point of regulation in the apoptotic pathways for diverse stresses.

Apoptotic cell death plays a central role in immune system development, in normal lymphocyte function, and in the progression of lymphoid neoplasia. B-lymphocytes have a propensity to undergo apoptosis as evidenced by their rapid death following the loss of cell-cell contacts, as well as their sensitivity to apoptosis induction following exposure to drugs or chemicals. Importantly, the role of appropriate signals within lymphoid organs in controlling the survival and apoptotic responses of B-lymphocytes has become increasingly apparent, and indicate a contribution of mitochondrial-dependent and independent pathways in B-cell apoptosis.

Cell lines derived from human Burkitt’s lymphoma (BL) have proven to be useful models of germinal center (GC) B-lymphocytes for investigating proliferative and apoptotic signals transduced by surface receptors, including the B-cell antigen-receptor (BCR), CD40, and CD95/Fas (9–19) as well as mechanisms of chemically induced cytotoxicity (18, 20–25). We previously identified a panel of BL cell lines that vary dramatically in their sensitivity to apoptosis induced by arsenite and to mitochondrial inhibitors (22, 23). The BL cell line ST486 shows sensitivity to BCR-mediated as well as chemically induced apoptosis, and it was selected here as a model for studying signaling pathways involved in regulation of antigen receptor and stress-induced cell death pathways.

Arsenite is a potent inducer of apoptosis in lymphoid cells. It is a ubiquitous environmental contaminant originating from geological and industrial sources with immunotoxic potential (26, 27). Furthermore, arsenite, in the form of arsenic trioxide, has emerged as a promising therapeutic agent for the treatment of acute promyelocytic leukemia (28). Arsenite is a prototypic chemical inducer of apoptosis that activates the c-Jun amino-terminal kinase (JNK) and the p38 pathways (23, 29, 30, 31) and acts via mitochondrial effectors of apoptosis (32). The differential sensitivity of BL cell lines to apoptosis induction by arsenite and other chemical agents can be attributed, at least in part, to expression of Bcl-2 proteins (22–25). This family of proteins is highly regulated throughout B-cell development.

*This w ork was supported by Grant R01ES010815 from the NIEHS, National Institutes of Health (to D. E. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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(33), but the signaling pathways that control the expression and activity of Bcl-2 and related proteins in B-cells are not completely known. Such information is important, because these proteins play a key role in regulating mitochondrial homeostasis and B-cell responses to stress-associated as well as BCR-mediated signals.

Depending on the stage of development, activation of the B-cell antigen receptor (BCR) has different cellular outcomes, including proliferation, growth arrest, apoptosis, and tolerance. BL cell lines serve as in vitro models for such BCR-mediated responses. Activation of the BCR by cross-linking of surface IgM leads to the induction of cell cycle arrest and apoptosis, whereas co-stimulation with CD40 and/or interleukin-4 results in rescue from IgM-mediated apoptosis. These processes parallel negative and positive selection of B-lymphocytes in vivo (9–19, 34–36). Although the ultimate outcome of BCR activation in BL cells is induction of apoptosis, the immediate signals are associated with survival/proliferative responses and include activation of the Ras/Raf/MEK/ERK and phosphoinositide 3-kinase (PI3K)/3-phosphoinositide-directed kinase-1 (PDK1)/protein kinase B/AKT pathways (AKT) (34, 37–42).

Among the downstream targets activated in response to surface IgM cross-linking is p90RSK ribosomal kinase (RSK) (38). Although first identified as a substrate of ERK, recent studies have shown that activation of RSK is dependent not only on MEK/ERK, but rather on the coordinated activity of MEK/ERK and PI3K/PDK1. ERK and PDK1 phosphorylate RSK at discrete sites, thereby activating the carboxyl- and amino-terminal domains (CTD and NTD), respectively (43–45). In addition, at least some of the downstream responses to IgM receptor activation involve members of the Bcl-2 family of proteins. Surface IgM engagement has been reported to induce cell death agonists Bax and Bik, whereas CD40 activation induces cell death antagonists BclX, and A1 (10, 11, 13–15). The pro-apoptotic protein, Bad, is a substrate of both AKT and RSK, which phosphorylate it at serine 136 and 112, respectively, subsequently blocking its pro-apoptotic activity (46–51). Phosphorylation of Bad has been reported following exposure of cells to cytokines and growth factors, but an involvement in IgM-mediated responses has yet to be described.

In the present study we tested the hypothesis that the rapid, coordinated activation of ERK and PI3K pathways following IgM cross-linking provides an initial survival signal that confers protection against the rapid induction of apoptosis by a broad range of stress factors. We found that surface IgM cross-linking in the ST486 cell line provides a window of protection against apoptosis induced by arsenite, multiple drugs that are associated with survival/proliferative responses and inhibition of mitochondrial activity (CTD and NTD), respectively (43, 44). Although first identified as a substrate of ERK, recent studies have shown that activation of RSK is dependent not only on MEK/ERK, but rather on the coordinated activity of MEK/ERK and PI3K/PDK1. ERK and PDK1 phosphorylate RSK at discrete sites, thereby activating the carboxyl- and amino-terminal domains (CTD and NTD), respectively (43–45). In addition, at least some of the downstream responses to IgM receptor activation involve members of the Bcl-2 family of proteins. Surface IgM engagement has been reported to induce cell death agonists Bax and Bik, whereas CD40 activation induces cell death antagonists BclX, and A1 (10, 11, 13–15). The pro-apoptotic protein, Bad, is a substrate of both AKT and RSK, which phosphorylate it at serine 136 and 112, respectively, subsequently blocking its pro-apoptotic activity (46–51). Phosphorylation of Bad has been reported following exposure of cells to cytokines and growth factors, but an involvement in IgM-mediated responses has yet to be described.

In the present study we tested the hypothesis that the rapid, coordinated activation of ERK and PI3K pathways following IgM cross-linking provides an initial survival signal that confers protection against the rapid induction of apoptosis by a broad range of stress factors. We found that surface IgM cross-linking in the ST486 cell line provides a window of protection against apoptosis induced by arsenite, multiple drugs that differ in their initial molecular targets in cells, and hyperthermia. This protection involves an inhibition of mitochondrial depolarization as well as caspase-9 cleavage, key points of regulation in chemically induced apoptotic pathways. Importantly, our study shows that the IgM-mediated protection requires activation of the ERK and the PI3K pathways. Moreover, these two signaling pathways converge upon a common downstream target, RSK, resulting in phosphorylation of the pro-apoptotic Bcl-2 family member, Bad.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Antibodies**—Sodium arsenite (NaAsO₂), carbonyl cyanide m-chlorophenylhydrazone (mCCCP), etoposide, and camptothecin were obtained from Sigma Chemical Co. (St Louis, MO). Sodium arsenite was dissolved in water, mCCCP in methanol, and all other chemicals in dimethyl sulfoxide.

Antibodies specific for total and/or phosphorylated p44/42 ERK, AKT threonine 389 and serine 378, RSK serine 380, Bad serine 112, Bad serine 136, PDK1 serine 241, and caspase-9 were purchased from Cell Signaling Inc. The antibody for poly(ADP-ribose) polymerase (PARP, AAB-258) was purchased from Stressgen, Inc. and for RSK serine 227 from Santa Cruz Biotechnology. Goat antifluorescence- purified F(ab')₂ fragment to human IgM was obtained from ICN/Cappel.

**Cell Lines and Culture Conditions and Chemical Treatment**—The BL cell lines ST486 and CA46 were obtained from ATCC, Rockville, MD. Both of the cell lines grow optimally and similarly in the same medium aminopterin, but have doubling times of ~24 h and are E2000-sensitive. The cell lines were cultured in medium RPMI 1640 (Invitrogen) supplemented with 15% fetal calf serum, penicillin-streptomycin, and 1-glutamine. Cells were grown at 37 °C, in 5% CO₂ and 95% humidity.

For all experiments, cultures were set up at a density of 0.4 ± 10⁵ cells/ml and allowed to grow for 24 h. For experiments with C6- cerebroide, cells were transferred to medium containing 1% FCS.) After appropriate pretreatment (i.e. 5 µg/ml anti-IgM or glycerol control) cells were plated into six-well plates, at 3 ml/well, and sodium arsenite or other chemicals were added at the indicated concentrations. At the designated times, cells were harvested for protein immunoblotting or flow analysis of morphological apoptosis as described below.

**Cytological Detection of Apoptosis and Necrosis with the Hoechst 33342/Propidium Iodide Assay**—The induction of apoptosis was analyzed using a double-fluorescence staining technique (53–55). The procedure allows simultaneous detection of plasma membrane integrity by dyes exclusion and apoptotic phenotypes by observing condensed, segregated chromatin in "live" cells. Briefly, cells were stained in 20 µg/ml propidium iodide (emitting red fluorescence) and 100 µg/ml Hoechst 33342 (emitting blue fluorescence) for 15 min, at 37 °C in the dark. The double fluorescence was detected with a Leitz Aristoplan microscope equipped with an epifluorescence system and a long-pass filter cube A. Dead cells emit red and live cells blue fluorescence. Apoptotic cells have a characteristic phenotype of condensed, segregated chromatin in intact but shrunken cells (fluorescing blue in early stages and red later on). The apoptotic phenotype was easy to detect and discriminate from necrotic cells, which were swollen, had irregular/damaged membranes, and were propidium iodide-positive. The chromatin was minimally condensed with some accumulation near the nuclear membrane. Typically, 200 cells were scored for each sample and classified as either necrotic, apoptotic, or normal/marrow, and data were subjected to statistical analysis as described below.

**Protein Immunoblotting**—Following chemical exposure for the specified period of time, cells (1 ml of culture) were collected, washed in phosphate-buffered saline, and solubilized in 50 µl of Laemmli sample buffer (65 µm Tris-CI, pH 6.8, 25% glycerol, 2% SDS, 0.1% bromphenol blue, and 5% β-mercaptoethanol). 10 µl of lysate (~4 × 10⁵ cells/sample) was subjected to SDS-PAGE in a 4 to 15% gradient gel. Gels were electrophoretically transferred to nitrocellulose membrane (Bio-Rad) in 25 mm Tris, pH 8.3, 192 mm glycine, 20% MeOH. For detection of phosphorylated kinases, membranes were first probed with antibodies specific for the phosphorylated forms then subsequently re-probed using antibodies that recognize the proteins independent of phosphorylation status as loading controls. Membranes were washed in TBS (20 mm Tris, 500 mm NaCl, pH 7.5) then blocked for 1 h in TBS containing 5% dried milk. Filters were then washed in TBS containing 0.1% Tween 20 then incubated overnight at 4 °C with primary antibody appropriately in TBS containing 5% bovine serum albumin. Filters were washed again and incubated with the second antibody-horseradish peroxidase conjugate. Detection was then performed using an enhanced chemiluminescent (ECL) system. Quantitation of the signal was performed using an Alpha Imager 2000 documentation and analysis system, equipped with AlphaEase version 3.2 software (Alpha Innotech Corp.).

**Detection of Mitochondrial Toxicant-induced Membrane Depolarization**—Mitochondrial toxicant-induced loss in ΔΨm was monitored at the single cell level using JC-1, a membrane potential-sensitive probe. JC-1 is taken up selectively by energized mitochondria and forms J-aggregates that emit red fluorescence upon excitation at 480 nm (55). Mitochondria with low ΔΨm (depolarized) take up little JC-1 and emit a low green fluorescence, indicative of the monomeric form of JC-1. A major advantage of the JC-1 assay is that the energy status of mitochondria can be observed in individual cells and a diagnosis made to determine whether 1) a majority of mitochondria are energized (red),
2) most mitochondria are depolarized (green), or 3) there is a mixture of energized and depolarized mitochondria in the same cell (partial depolarization) (56).

Cultures were seeded as described above, incubated for 24 h, and then exposed to graded concentrations of arsenite or mCCCP at the indicated concentrations. At each of the respective time points, 5 μl of JC-1 stain (from a 400 μM stock in 59x663 FIG. 1.

Cultures of ST486 cells were established at a density of 4 × 10⁵ cells/ml and grown for 24 h. IgM antibody at 5 μg/ml (+anti-IgM) or glycerol (−anti-IgM) was added 20 min prior to the addition of arsenite, at the indicated concentrations. At 4 h following chemical addition, aliquots were taken, and cells were lysed and subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting using PARP antibody that recognizes the 113-kDa uncleaved protein and the 85-kDa cleavage fragment. The percentage of cleaved PARP, as determined by densitometry, is indicated below each lane. The results are representative of a minimum of three independent experiments.

2) most mitochondria are depolarized (green), or 3) there is a mixture of energized and depolarized mitochondria in the same cell (partial depolarization) (56).

Cultures were seeded as described above, incubated for 24 h, and then exposed to graded concentrations of arsenite or mCCCP at the indicated concentrations. Cell cultures were sampled after 2, 4, and 8 h of chemical exposure to study the kinetics of loss of Δψm. At each of the respective time points, 5 μl of JC-1 stain (from a 400 μM stock in

Fig. 2. Surface IgM cross-linking provides transient protection against the induction of morphological apoptosis, as well as PARP and caspase-9 cleavage in ST486 cells that is independent of the order of addition of antibody or arsenite. A, cultures of ST486 cells were treated with IgM antibody (IgM, solid bars), 20 μM sodium arsenite (AS, shaded bars), IgM antibody plus arsenite (IgM + AS, striped bars), or left untreated (control, open bars). In those cultures subjected to IgM cross-linking, antibody was added 20 min prior to the addition of arsenite. After incubation for the times indicated, aliquots were taken and morphological apoptosis was quantified using Hoechst 33342/propidium iodide staining as described under “Experimental Procedures.” The mean values and S.E. were established from three independent experiments. Statistically significant differences (p < 0.05) between samples treated with arsenite alone and those treated with IgM antibody prior to arsenite addition were found at 4, 6, and 8 h. Cultures treated with arsenite alone were significantly different from control cultures for all time points, whereas cultures treated with anti-IgM plus arsenite were significantly different from controls at 6, 8, and 24 h. B, cultures of ST486 cells were treated with 20 μM sodium arsenite (AS) or IgM antibody plus arsenite (anti-IgM + AS) as described above and sampled at the indicated times. Cells were lysed and subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting using PARP antibody or an antibody to caspase-9 (CSP 9), which detects the 47-kDa parental protein, plus 37- and 35-kDa cleavage products. The percentage of cleaved PARP and caspase-9, as determined by densitometry, is indicated below each lane.

C, cultures of ST486 cells were treated with 20 μM arsenite for a total of 4 h. IgM antibody was added as follows: no antibody (−IgM), antibody 20 min prior to chemical addition (20m), or 30 min, 1 h, 2 h, or 3 h after chemical addition (+30m, +1h, +2h, +3h, respectively). After a total of 4 h of chemical exposure, lysates were made and subjected to protein immunoblotting with PARP antibody.
measured as early as 4 h following exposure to 20 μM arsenite (Fig. 2A, striped bars) compared with those exposed to arsenite alone (Fig. 2A, shaded bars). A similar to PARP cleavage, morphological changes characteristic of apoptosis were detected as early as 4 h following exposure to 20 μM arsenite (Fig. 2A, striped bars), whereas apoptosis induction following surface IgM cross-linking was detected only at 24 h (Fig. 2A, black bars).

Despite the rapid induction of apoptosis by arsenite, cross-linking of surface IgM prior to addition of arsenite almost completely suppressed arsenite-induced PARP cleavage at 4 h (Fig. 1, \(-anti\)-IgM compared with \(+anti\)-IgM). IgM-mediated suppression of arsenite-induced apoptosis was confirmed by the morphological assay (Fig. 2A). Significantly fewer apoptotic cells were detected in cultures treated with IgM-antibody plus arsenite (striped bars) compared to those exposed to arsenite alone (shaded bars) at 4 h, and reduced levels of apoptosis due to IgM cross-linking were sustained for a period of at least 8 h. Consistent with morphological apoptosis, reduced levels of PARP cleavage were detected over several hours, with only a modest increase appearing at the later time points of 6 and 8 h (Fig. 2B, PARP, AS compared with AS \(+anti\)-IgM).

Caspapectase-9 is a proximal caspase in the apoptotic pathway. It is activated upon release of cytochrome c from mitochondria, an event that is central to the execution of the apoptotic program and common to a broad spectrum of apoptotic stimuli. The activation of procaspase-9 involves the autocatalytic cleavage of the full-length 47-kDa protein to generate fragments of 35 and 17 kDa. Among the substrates cleaved by activated caspase-9, PARP is a key player in the apoptotic process, and its cleavage is a hallmark of apoptosis.

**RESULTS**

**Activation of the Surface IgM Receptor Protects ST486 Cells from Arsenite-induced Apoptosis during an 8-h Period—**The ST486 cell line is a model system for time-dependent IgM-mediated apoptosis (17–19) as well as apoptosis induction by the arsenite and other chemicals (22, 23). However, the kinetics of apoptosis induction by arsenite treatment compared with surface IgM receptor activation differed markedly. Sodium arsenite-induced apoptosis in ST486 cells as early as 4 h following exposure as evidenced by levels of PARP cleavage of over 50% at this time point (Fig. 1, \(-anti\)-IgM). In contrast, no PARP cleavage was detected at 4 h following cross-linking of surface IgM (Fig. 1, \(+anti\)-IgM, 0 μM AS). Similar to PARP cleavage, morphological changes characteristic of apoptosis were detected as early as 4 h following exposure to 20 μM arsenite (Fig. 2A, shaded bars), whereas apoptosis induction following surface IgM cross-linking was detected only at 24 h (Fig. 2A, black bars).

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**Fig. 3. Cross-linking of surface IgM delays the loss of Δψm following exposure to arsenite but not to mCCCP and fails to protect against mCCCP-induced apoptosis.** A, cultures of ST486 cells were treated as follows: IgM antibody alone (IgM), 20 μM arsenite (AS), IgM antibody plus 20 μM arsenite (IgM + AS), 5 μM mCCCP (CCP), or IgM antibody plus 5 μM mCCCP (CCP + IgM). Aliquots of cultures were taken at the indicated times, and loss of Δψm was determined using the membrane potential-sensitive JC-1 fluorochrome as described under “Experimental Procedures.” The graph shows the percentage of cells remaining polarized (normal membrane potential). The mean values and S.E. were established from three independent experiments. The values for cultures treated with IgM plus arsenite were significantly higher than those for cultures treated with arsenite alone at 2 and 4 h, whereas the values for cultures treated with anti-IgM plus mCCCP were significantly lower than those for cultures treated with mCCCP alone at 2, 4, and 8 h (p < 0.05). B, cultures of ST486 cells were treated with IgM antibody (+anti-IgM) or glycerol (–anti-IgM) for 20 min prior to the addition of 2, 5, or 10 μM mCCCP (CCP) or 20 μM arsenite (AS). At 4 h following chemical addition, aliquots were taken, and cells were lysed and subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting using PARP antibody that recognizes the 113-kDa uncleaved protein and the 85-kDa cleavage fragment. The percentage of cleaved PARP and caspase-9, as determined by densitometry, is indicated below each lane. C, cultures of ST486 cells were treated as described for B, and aliquots were collected and subjected to analysis of morphological apoptosis at the indicated times. The treatments are: untreated (control, open bars), IgM antibody (IgM, solid bars), IgM antibody plus mCCCP (IgM + CCP, shaded bars), IgM antibody plus mCCCP (IgM + CCP, striped bars). The mean values and S.E. were established from three independent experiments. Statistically significant differences (p < 0.05) between samples treated with mCCCP alone and those treated with anti-IgM plus mCCCP were found at 6 and 8 h, with no statistical difference revealed at 4 h.
caspase-9 is caspase-3. In addition to autocatalytic cleavage, procaspase-9 can be cleaved by mitochondrial-associated/activated caspase-3 to generate a 37-kDa fragment, which contributes to the amplification of caspase-9 processing. IgM cross-linking delayed the generation of both the 35- and 37-kDa cleavage products of procaspase-9 in a manner that paralleled the delay in PARP cleavage over the 8-h period (Fig. 2B).

In the experiments described above, IgM antibody was added 20 min prior to the addition of arsenite. However, IgM cross-linking also blocked PARP cleavage when added up to 2 h after the chemical (Fig. 2C). Arsenite itself is a potent inducer of stress kinases, including p38 and JNK in many cell types, including those derived from BL. We have previously shown that activation of stress kinases by arsenite occurs rapidly in ST486 cells, reaching maximal levels within 1 h of chemical addition (23). Thus, IgM cross-linking blocks arsenite-induced apoptosis after the immediate signaling events induced by arsenite, such as stress-kinase activation, but prior to the cleavage of pro-caspase-9. Together, these data suggest the possibility that mitochondria may be a target of IgM-mediated protection.

Cross-linking of the Surface IgM Receptor Inhibits Mitochondrial Depolarization Induced by Arsenite—Loss of $\Delta \psi_{\text{m}}$ is an important marker of mitochondrial involvement in the apoptotic pathway. This event is often accompanied by permeability transition and release of cytochrome c leading to caspase-9 cleavage/activation. We used the mitochondrial membrane-sensitive fluorochrome JC-1 to measure $\Delta \psi_{\text{m}}$, and to determine if IgM cross-linking inhibited the depolarization of mitochondria by arsenite compared with mClCCP (Fig. 3A). This latter agent directly affects mitochondrial function, causing a rapid and extensive uncoupling of oxidative phosphorylation leading to collapse of $\Delta \psi_{\text{m}}$ (53, 54).

A progressive decline in $\Delta \psi_{\text{m}}$ was detected in arsenite-treated cells (AS) at 2–8 h of chemical exposure. Prior cross-linking of surface IgM (AS plus IgM) inhibited the loss of $\Delta \psi_{\text{m}}$ over a 4-h period in a manner that paralleled the delay of caspase-9 and PARP cleavage and morphological apoptosis. In contrast, IgM cross-linking failed to protect against, and even augmented, loss of $\Delta \psi_{\text{m}}$ induced by 5 $\mu$m mClCCP compared with CCP plus IgM). Further analysis revealed that IgM cross-linking provided only minimal protection against mClCCP-induced PARP or caspase-9 cleavage (Fig. 3B) that was limited to the lowest concentration of 2 $\mu$m. No protection was observed for 5 or 10 $\mu$m mClCCP, concentrations that gave levels of PARP cleavage similar to 20 $\mu$m arsenite. Analysis of morphological apoptosis induced by 5 $\mu$m mClCCP over a period of 8 h revealed that IgM cross-linking did not protect, but actually potentiated, apoptosis induced by this toxicant (Fig. 3C).

The ability of IgM cross-linking to transiently inhibit mitochondrial depolarization by arsenite, but not by mClCCP, suggests that protection may be associated with a specific mitochondrial target involved in apoptosis, as opposed to a general stabilization of mitochondrial function. However, such an effect should not be limited to arsenite but should extend broadly to other agents that activate a mitochondrial pathway for apoptosis induction. Thus, we investigated whether IgM cross-linking conferred protection to apoptosis induced by other chemicals that differ in their initial molecular targets in cells as well by potential endogenous effectors of apoptosis (Fig. 4). Arsenite interacts with sulphhydryl groups in proteins and can...
generate reactive oxygen species. Other chemicals used were camptothecin, an inhibitor of DNA topoisomerase I; etoposide, an inhibitor of DNA topoisomerase II; and the protein synthesis inhibitor, cycloheximide (Fig. 4A). We also examined the effect of anti-IgM pretreatment on apoptosis induced by hyperthermia (Fig. 4, B and D) and by C6-ceramide (Fig. 4, C and D). Ceramide is generated by the hydrolysis of complex sphingolipids and has emerged as an important coordinator of cellular stress responses in a number of cell types, including immune cells (reviewed in Ref. 57). It is considered a second messenger of signaling pathways and is induced by a variety of exogenous and endogenous stresses, including chemicals, bacterial endotoxin, hyperthermia, and hypoxia (57–59). All of the above treatments induced apoptosis in ST486 cells within 4–6 h of exposure. Prior cross-linking of surface IgM substantially inhibited PARP cleavage and apoptosis induction.

**Contribution of the MEK/ERK and PI3K/PDK1/AKT Pathways to IgM-mediated Protection against Apoptosis.** A number of signaling pathways are activated as a consequence of engagement of the surface IgM receptor, including MEK/ERK and PI3K/PDK1/AKT. However, their role in regulating cellular responses to stress is unclear. We examined the potential contribution of these pathways in IgM-mediated protection against apoptosis. Consistent with published studies, we found that IgM cross-linking rapidly induced the phosphorylation of AKT and ERK in ST486 cells (Fig. 5A). Although phosphorylation was sustained over the first 4 h of treatment, the levels of phosphorylated kinases declined somewhat from 4 to 8 h (Fig. 5B), particularly for AKT. Exposure of cells to arsenite accelerated the loss in signal for both kinases. It should be noted that, although the two isoforms of ERK, p42 and p44, are not completely resolved here, they showed similar activation profiles in all experiments. In addition, although the results shown for AKT were obtained using an antibody specific for phosphorylation at threonine 308, similar results were obtained using a serine 473-specific antibody.

Both of these signaling pathways are well documented in their ability to promote proliferative and/or survival responses in cells. Thus, we assessed the potential contribution of each pathway to IgM receptor-mediated protection using specific chemical inhibitors (Fig. 6). The inhibitors used were: LY294002 (LY), an inhibitor of PI3K that is upstream of AKT; U0126, an inhibitor of MEK1/2, the kinase immediately upstream of ERK1/2; AKTi5, a phosphatidylinositol ether analog that binds the pleckstrin homology domain of AKT and inhibits its phosphorylation (52); or rapamycin, an inhibitor of the mTOR, which is a downstream substrate of AKT. Each of these agents effectively blocked IgM-mediated phosphorylation of the expected target kinases the concentrations used (Figs. 6C and 8A).

The inhibitors did not induce apoptosis induction or PARP cleavage when added alone to cells (Fig. 6, −AS/−IgM) or when added to cultures treated with either IgM antibody (−AS/+anti-IgM) or arsenite (+AS/−anti-IgM) alone for the duration of the experimental period (i.e. a total of 6 h of treatment). However, it should be noted that long term exposure (i.e. 24 h) to the high concentration of LY, U0126, or AKTi5 did induce some apoptosis.

As shown previously, IgM cross-linking effectively blocked arsenite-induced PARP cleavage at 4 h (Fig. 6, +AS/−anti-IgM) compared with +AS/+anti-IgM for 0 μM inhibitor). Prior exposure of cells to graded concentrations of LY or U0126 (Fig. 6A) resulted in a partial reversal of the protective effect of IgM cross-linking, whereas AKTi5 and rapamycin did not (Fig. 6B). The later two inhibitors failed to reverse IgM-mediated protection at inhibitor concentrations as high as 40 μM and 40 nM,
respectively (data not shown). In addition, wortmannin, another PI3K inhibitor that is structurally unrelated to LY, acted in a similar manner (data not shown). We were unable to determine the effects of simultaneously blocking both the ERK and PI3K pathways, because the combined treatment of ST486 with U0126 plus LY (or wortmannin) resulted in substantial levels of apoptosis within the few hours required for the experiment. However, treatment of cells with phorbol ester, a potent activator of ERK, also resulted in protection against arsenite-induced apoptosis that was inhibitable by U0126 (data not shown). Together, these data suggest the independent contributions of the MEK/ERK pathway, as well as components of the PI3K pathway, separate from AKT or mTOR, to cell survival signals that are initially activated by surface IgM cross-linking.

MEKK/ERK and PI3K-mediated Signals Converge on Phosphorylation of Bad—Activation of the MEK/ERK and PI3K/AKT pathways have several possible outcomes in cells, including modulating of mitochondrial resistance to apoptotic stimuli. Therefore, we examined the effect of IgM cross-linking on Bad, a pro-apoptotic member of the Bcl-2 family of proteins that promotes the release of cytochrome c/caspase-9 activation and a potential downstream target of both of these signaling pathways. The pro-apoptotic activity of Bad is mediated by its ability to interact with, and inhibit, the anti-apoptotic activity of Bcl-2 and Bcl-X<sub>L</sub>. Phosphorylation of Bad at serine 112 and/or serine 136 results in its release from these proteins, thereby facilitating their anti-apoptotic function. Serine 112 is a known, although indirect, substrate of ERK, whereas serine 136 is a substrate of AKT (46–51). We found that cross-linking of surface IgM in ST486 cells rapidly induced phosphorylation of Bad at serine 112 (Fig. 7A). In contrast, we were unable to detect phosphorylation of Bad at serine 136 (data not shown). Together, these data suggest the independent contributions of the MEK/ERK pathway, as well as components of the PI3K pathway, separate from AKT or mTOR, to cell survival signals that are initially activated by surface IgM cross-linking.

**FIG. 6.** IgM-mediated protection against arsenite-induced apoptosis is blocked by the MEKK1/2 inhibitor U0126 and the PI3K inhibitor LY294002. Cultures of ST486 cells were treated with (A) LY or U0126 or (B) AKTi5 or rapamycin (RAP) at the indicated concentrations for 1 h. After that time, either anti-IgM (+anti-IgM) or glycerol (−anti-IgM) was added to the appropriate cultures for an additional 20 min. Cultures were then exposed to 20 μM arsenite (−AS or +AS), as indicated. At 4 h following addition of arsenite, lysates were made and subjected to immunoblotting with PARP antibody. The percentage of cleaved PARP, as determined by densitometry, is indicated below each lane. C, cultures of ST486 cells were treated with 0, 10, 20, or 40 μM AKTi5 for 1 h prior to the addition of anti-IgM as indicated. After an additional 1-h incubation, protein lysates were made and immunoblotted with antibodies that recognize AKT and phospho-AKT. The percentage of signal for phospho-AKT in anti-IgM plus AKTi5-treated cultures compared with the culture treated with anti-IgM alone is indicated below each lane.

**FIG. 7.** Anti-IgM treatment induces phosphorylation of Bad at serine 112 that can be inhibited by U0126 and LY294002. A, cultures of ST486 and CA46 cells were treated with anti-IgM, or left untreated, as indicated for 1 h. Lysates were made and immunoblotted using antibodies specific for Bad phosphorylated at serine 112 or with antibody that recognizes Bad regardless of phosphorylation state. B, cultures of ST486 cells were treated with LY, U0126, or rapamycin (RAP) at the indicated concentrations for 1 h prior to the addition of anti-IgM. Lysates were made 1 h after antibody addition and subjected to immunoblotting with an antibody specific for Bad serine 112.
RSK is a substrate of ERK responsible for phosphorylation of numerous protein targets, including Bad at serine 112 (50, 60). In addition, recent studies show that MEKK/ERK and PI3K/PDK1 together contribute to the activation of RSK1 and 2 by a series of independent phosphorylations (43–45). The regulation of RSK is complex and is described in more detail under “Discussion.” Briefly, ERK-dependent phosphorylations occur at sites in its CTD and linker region, including serines 380/386, whereas PDK1 phosphorylates serines 221/227 in the NTD. These multiple phosphorylations confer full activity to the amino-terminal kinase. Thus, we predicted that U0126 and LY would inhibit phosphorylation of the critical sites on RSK that are dependent upon the activity of MEK/ERK (i.e. serines 380/386) and PI3K/PDK1 (serines 221/227), respectively, thereby blocking the phosphorylation of Bad at serine 112. (It should be noted that the phospho-specific antibodies used in these experiments recognize both isoforms of RSK, 1 and 2.)

Inhibition of the MEK/ERK pathway using U0126 blocked the phosphorylation of RSK serine 380/386 following IgM cross-linking (Fig. 8A). Consistent with published studies on other cell types, PDK1 was constitutively phosphorylated in ST486 cells, as was its target in RSK, serine 221/227, although we detected a small, but consistent increase in the intensity of phospho-serine 221/227 following IgM cross-linking. Importantly, treatment with LY effectively blocked the phosphorylation of this site, in control and in antibody-treated cultures, indicating a requirement for PI3K. Rapamycin had no effect on phosphorylation of either site, consistent with its failure to inhibit Bad phosphorylation or reverse IgM-mediated protection against apoptosis (Fig. 8B).

**DISCUSSION**

Our study shows, for the first time, that cross-linking of surface IgM initially confers a survival signal that protects ST486 cells against the induction of apoptosis for a period of several hours following BCR engagement. This protective effect extends to a variety of chemicals with different molecular targets, including inhibitors of transcription and protein synthesis, as well as to hyperthermia and ceramide. Furthermore, BCR-mediated protection targets mitochondrial effectors of apoptosis, blocking the loss of Δψm and cleavage of caspase-9.

GC B-lymphocytes rapidly undergo apoptosis when deprived of appropriate cell-cell contact or when exposed to chemical/physical stress. Loss of signals derived primarily from follicular dendritic cells results in the rapid degradation of the Fas-associated death domain (FADD)-inhibitory protein, FLIP, and induction of apoptosis by activation of the FAS/FADD/caspase-8 pathway (2–5). Association of GC B-cells with follicular dendritic cells inhibits this response by preventing the loss of FLIP. Importantly, this is a type I Fas pathway, independent of mitochondrial effectors of apoptosis, and is therefore unaffected by the expression of members of the mitochondrial-associated Bcl-2 family of proteins.

However, GC B-cells are also vulnerable to many pro-apoptotic signals that exert effects through a mitochondrial route. Apoptosis induced by engagement of antigen receptor during the process of negative selection against self-reactive B-cells requires mitochondrial signals (61–65). In addition, conditions that may alter the GC microenvironment, including endogenous stresses that occur during infection (i.e. hyperthermia, anoxia, and septic shock) as well as exogenous stresses resulting from drug/chemical exposure (66–68), also induce apoptosis in B-cells. In contrast to the Fas pathway described above, stress-associated stimuli activate mitochondrial effectors and that can be modulated by Bcl-2 and its related proteins (22–25).

Survival factors within the GC, such as CD40, can protect mitochondria by up-regulation of Bcl-2-related proteins, A1 and BclX, via the transcriptional trans-activator NFκB (13, 14). Because these responses involve new protein synthesis, they are postulated to contribute to long term B-cell survival. Our results suggest an additional, but presently overlooked, role for the BCR that specifically modulates the sensitivity of B-cells to apoptotic stimuli. Although at present we cannot exclude an interaction between BCR-mediated signals and the Fas pathway, we have clear evidence that activation of surface IgM can influence survival of ST486 cells by stabilizing mitochondria. The BCR-mediated response is immediate and, unlike CD40 protection, does not require new protein synthesis, as evidenced by the surface IgM-mediated protection against cycloheximide. Thus, the transient protection afforded by BCR engagement may ensure a window of protection during which a decision can be made for either selection/proliferation or apoptosis of B-cells after encountering antigen.
We found that the protective effect of IgM receptor activation against chemically induced apoptosis was mediated by PI3K-associated signals and by the MEK/ERK pathway. Pharmacological inhibition of each pathway using LY or U0126, respectively, partially reversed IgM-mediated suppression of arsenite-induced apoptosis. Our data also showed a gradual reduction in the phosphorylation of the target kinases activated by surface IgM cross-linking that was accelerated in the presence of arsenite and paralleled the decay in suppression of apoptosis. However, we found that inhibiting AKT, or downstream target mTOR, failed to block IgM-mediated protection against apoptosis, thus suggesting that the required PI3K-mediated signals are independent of AKT and/or mTOR.

The importance of PI3K activation has clearly been demonstrated in B-lymphocyte development and functions in both in vivo and in vitro systems. PI3K-knockout mice are defective in B-cell responses and fail to develop germinal centers. Surface IgM cross-linking of B-lymphoma cell lines induced a transient activation of p70S6 kinase, a downstream target of AKT/mTOR, as well as an initial increase in c-Myc protein (40), both of which are associated with cell growth and survival. Inhibition of PI3K with LY, or transfection of cells with dominant-negative PI3K, acted synergistically with IgM cross-linking to enhance growth inhibition and apoptosis induction, whereas transfection with constitutively active PI3K abrogated the cytotoxic effects of IgM cross-linking. Similarly, AKT has been shown to be essential for proliferation of lymphoid cells (70). However, consequences of PI3K activation in B-cells in all of these cases are attributed to the PI3K/AKT pathway and subsequent downstream targets of AKT that effect gene expression. We have identified a target of PI3K that is activated by engagement of surface IgM but independent of AKT, specifically, RSK/Bad, which influences B-cell survival by inducing a rapid and complete protection of mitochondria.

Mitochondrial changes, in particular loss of Δψ, leading to release of cytochrome c and subsequent caspase-9 cleavage, are associated with apoptosis induced by a variety of stimuli. We found that IgM cross-linking transiently inhibited the loss of Δψ in arsenite-treated cells with kinetics that paralleled the delay in caspase-9 and PARP cleavage. Such specific effects suggest the regulation of mitochondrial sensitivity by IgM cross-linking, possibly mediated by members of the Bcl-2 family of proteins.

ST486 cells have relatively low levels of the anti-apoptotic Bcl-2 protein and relatively high levels of the pro-apoptotic protein Bax (22, 24). Studies show that IgM receptor- and chemically induced apoptosis may be mediated, in part, by changes in the levels or ratio of Bcl-2 to Bax. We did not observe a change in either of these proteins upon arsenite exposure or IgM receptor cross-linking (data not shown). However, we found that IgM cross-linking did induce a rapid phosphorylation of BAD, a pro-apoptotic Bcl-family member. BAD forms an initial increase in c-Myc protein (40), both of these proteins upon arsenite exposure or IgM receptor cross-linking (data not shown). However, we found that IgM cross-linking did induce a rapid phosphorylation of BAD, a pro-apoptotic Bcl-family member. BAD forms an association and results in its sequestration in the cytosol by 14-3-3 protein. Much of the published literature demonstrates that the MEK/ERK pathway mediates the phosphorylation of serine 112, whereas the phosphorylation of serine 136 is mediated by the PI3K/AKT pathway, either directly by AKT, or by the downstream target p70S6 kinase (46–51). However, our results are most consistent with a model in which both pathways independently contribute to the phosphorylation of BAD at serine 112 and invoke RSK as a point of convergence. RSK is a major target activated by the MEK/ERK pathway following exposure of cells to growth factors, insulin, and other stimuli (73) and by engagement of the surface IgM receptor (38). It has been invoked as the kinase primarily responsible for phosphorylation of Bad at serine 112 in response to ERK activation (48, 49, 60). The regulation of RSK is complex, but data suggest the involvement of both MEK/ERK as well as PI3K/PDK1 (43–45). Current models indicate the requirement for phosphorylation of at least two sites by ERK located in the carboxy-terminal domain and the linker region. These phosphorylations subsequently activate the carboxy-terminal kinase leading to autophosphorylation of serine 380 or 386 in the linker region of RSK 1/2. This event generates a docking site for PDK1, which phosphorylates serines 221/227 and activates the amino-terminal kinase of RSK. Full activity of RSK is dependent on all of these phosphorylation events. Our data are consistent with this model in that inhibition of MEK/ERK with U0126 and inhibition of PI3K with LY specifically blocked phosphorylation of serine 380/386 and serine 221/227, respectively, and, consequently, the ability of RSK to phosphorylate BAD at serine 112 following surface IgM cross-linking.

Our result showing phosphorylation of BAD as an early response to BCR activation is consistent with a central role for members of this protein family in regulating apoptotic responses of B-cells. Studies show that other components of the germinal center microenvironment, such as CD40 ligation or association with follicular dendritic cells, not only modulate normal B-cell responses but also can protect them from apoptosis induced by antineoplastic drugs (5, 74). Our study suggests not only a role for BCR in modulation of normal B-cell responses described above but also the potential, along with co-stimulatory signals, to influence the outcome of antineoplastic or other forms of immunotherapy.

Acknowledgments—We thank Linda Hovanc for technical assistance, Dr. Mark Roberson for helpful discussions and advice, and Dr. Allan Eaglesham for editorial assistance.

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