CD40 Signaling in B Cells Regulates the Expression of the Pim-1 Kinase Via the NF-κB Pathway[1]

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The ability of CD40 signaling to regulate B cell growth, survival, differentiation, and Ig class switching involves many changes in gene expression. Using cDNA expression arrays and Northern blotting, we found that CD40 signaling increased the mRNA levels for *pim-1*, a protooncogene that encodes a serine/threonine protein kinase. Subsequent experiments showed that CD40 engagement also increased both Pim-1 protein levels and Pim-1 kinase activity in B cells. We then investigated the signaling pathways by which CD40 regulates Pim-1 expression and found that CD40 up-regulates Pim-1 primarily via the activation of NF-κB. Inhibiting the activation of NF-κB, either by treating cells with a chemical inhibitor, BAY11-7082, or by inducibly expressing a superrepressor form of IκBα, significantly impaired the ability of CD40 to increase Pim-1 protein levels. Because Pim-1 expression is associated with cell proliferation and survival, we asked whether this correlated with the ability of CD40 signaling to prevent anti-IgM-induced growth arrest in the WEHI-231 murine B cell line, a model for Ag-induced clonal deletion. We found that the anti-IgM-induced growth arrest in WEHI-231 cells correlated with a substantial decrease in Pim-1 levels. In contrast, culturing WEHI-231 cells with either anti-CD40 Abs or with the B cell mitogen LPS, both of which prevent the anti-IgM-induced growth arrest, also prevented the rapid decline in Pim-1 levels. This suggests that Pim-1 could regulate the survival and proliferation of B cells. ***The Journal of Immunology*****, 2002, 168: 744–754.**

The TNFR family member CD40 plays a central role in T cell-dependent B cell activation (1, 2). The CD40 ligand (CD40L)[3] is expressed on activated T cells and delivers costimulatory signals that prevent B cell receptor (BCR)-induced apoptosis or anergy (3, 4). CD40 signaling also promotes the proliferation of activated B cells, the survival of germinal center B cells, Ig secretion, memory cell formation, and Ig isotype switching (5–12). The essential role of the CD40/CD40L interaction in the development of humoral immunity is illustrated by the defects observed in hyperIgM syndrome, an immunodeficiency disease due to loss-of-function mutations in CD40L. B cells from patients with this disease fail to undergo Ig class switching and do not form germinal centers where somatic hypermutation of Ig-variable genes can occur (13, 14). Similarly, mice lacking CD40 or CD40L are unable to generate secondary humoral immune responses to T cell-dependent Ags (15, 16).

CD40 signals via TNFR-associated factors (TRAFs), adaptor proteins that bind to the CD40 cytoplasmic domain (17, 18). TRAF2, TRAF3, TRAF5, and TRAF6 bind to CD40 and mediate the activation of multiple signaling pathways that regulate transcription factors. CD40 has been shown to activate the NF-κB transcription factor (19, 20) as well as the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein (MAP) kinases (21–25), which phosphorylate and activate transcription factors. ERK phosphorylates and activate Ets domain-containing transcription factors while JNK and p38 phosphorylate and activate the c-Jun, activating transcription factor-2, CHOP, and MEF2C transcription factors (26). CD40 also activates MAPKAP kinase-2 (25), a downstream target of p38 that phosphorylates and activates the CREB transcription factor (27). In addition to these signaling pathways, phosphatidylinositol 3-kinase (PI3K) has also been implicated in CD40 signaling (28, 29). The specific roles of NF-κB, ERK, JNK, p38, MAPKAP kinase-2, and PI3K in mediating responses to CD40 engagement have not been fully delineated.

The various transcription factors that are regulated by CD40 signaling pathways presumably modulate the expression of genes that control B cell survival and proliferation, Ig class switching, and the differentiation of B cells into Ab-secreting cells and memory cells. These complex cellular responses to CD40 engagement are likely to involve many changes in gene expression. For example, CD40 signaling has been shown to regulate the expression of multiple genes encoding antiapoptotic proteins (Bcl-x_L, A1, A20, cIAP2, c-Myc (30–34)) as well as a variety of genes encoding cell surface proteins that allow B cells to communicate with other cells of the immune system (CD40, CD23, LFA-1, ICAM-1, Fas, B7.1, B7.2 (7, 33, 35–39)). However, it is likely that many other CD40-regulated genes also contribute to the ability of CD40 to promote B cell survival, activation, proliferation, and differentiation.

To identify additional CD40-responsive genes that might regulate B cell survival and activation, we used Clontech Atlas gene

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Received for publication July 30, 2001. Accepted for publication November 8, 2001.

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[1] This work was supported by grants from the Arthritis Society of Canada (to M.R.G.), the National Institutes of Health (Grants AI28847 and CA66570 to G.A.B.), the U.S. Department of Veterans Affairs (Merit Review Award 383 to G.A.B.), and the U.S. Department of Agriculture (Grant 91-37206-6867 to N.S.M.).

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[3] Abbreviations used in this paper: CD40L, CD40 ligand; BCR, B cell receptor; TRAF, TNFR-associated factor; MAP, mitogen-activated protein; PI3K, phosphatidylinositol 3-kinase; IPTG, isopropyl β-D-thiogalactopyranoside; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase.

0022-1767/02/$02.00

array filters (Clontech Laboratories, Palo Alto, CA) to monitor the expression of >500 genes that are known to be involved in apoptosis, proliferation, differentiation, and cell-cell communication. In particular, we wished to identify genes that are rapidly induced by CD40 signaling and which may play a key role in determining whether B cells survive and proliferate as opposed to undergoing apoptosis. Therefore, we used as our model system the WEHI-231 murine B cell line in which CD40 signaling prevents anti-IgM-induced growth arrest and apoptosis (4, 40) by delivering both survival signals and proliferative signals (2, 41).

Using this gene array analysis, we identified the *pim-1* gene as a target of CD40 signaling. *pim-1* is a protooncogene that encodes a serine-threonine kinase (42, 43) whose expression is associated with the survival and proliferation of hematopoietic cells. *pim-1* expression is induced by a variety of cytokines, growth factors, and mitogens including IL-2, IL-3, IL-6, IL-9, IL-12, IL-15, erythropoietin, GM-CSF, G-CSF, IFNs α and γ, prolactin, Con A, PMA, and anti-CD3 Abs (44–52). Moreover, in the FDCP1 myeloid cell line, constitutive expression of an exogenous *pim-1* gene can inhibit apoptosis caused by cytokine deprivation, DNA-damaging agents, and Bax expression (53–55). In normal cells, the expression of a *pim-1* transgene can suppress the spontaneous ex vivo apoptosis of peripheral T cells as well as the dexamethasone-induced apoptosis of thymocytes (56). *pim-1* is a weak oncogene by itself, but it cooperates with c-*myc* to cause pre-B cell lymphomas (57–59). Because CD40 signaling increases c-*myc* expression (34), the concomitant up-regulation of *pim-1* expression could contribute to the ability of CD40 to promote B cell survival and proliferation. Therefore, we investigated the regulation of Pim-1 levels in B cells and its relationship to B cell survival and proliferation.

In this report, we show that CD40 signaling increases the levels of Pim-1 protein and Pim-1 kinase activity in B cells. Moreover, we found that CD40 regulates Pim-1 levels via the activation of NF-κB. We also show that Pim-1 levels in B cells are regulated by both BCR engagement and LPS stimulation and that signals emanating from the BCR, CD40, and the LPS receptor complex are integrated at the level of Pim-1. Finally, we show that there is a correlation between Pim-1 protein levels and the survival and proliferation of WEHI-231 B lymphoma cells.

Materials and Methods

Materials

The 1C10 anti-murine CD40 mAb (60) was purified from hybridoma supernatants using a protein A-Sepharose column. Goat anti-mouse IgM (μ-chain-specific) Abs were purchased from BIO/CAN Scientific (Mississauga, Ontario, Canada). A rabbit Ab raised against amino acids 1–37 of human p33 Pim-1 (anti-Pim-1-NT, a gift from Dr. S. Pelech (University of British Columbia, Vancouver, Canada) (61)) was used for immunoblotting while a rabbit Ab raised against a GST-human Pim-1 fusion protein (N. S. Magnuson, unpublished data) was used for in vitro kinase assays. Mouse anti-actin Abs were from Sigma-Aldrich (St. Louis, MO). A rabbit Ab that specifically recognizes IκBα that is phosphorylated on serine 32 (anti-phospho-IκBα) was purchased from New England Biolabs (Beverly, MA) while the goat anti-RelA Ab (Ab c-20) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). *Escherichia coli* 0111:B4 LPS was obtained from Calbiochem (La Jolla, CA), cycloheximide was from Sigma-Aldrich, and the NF-κB inhibitor BAY11-7082 (62, 63) was from Biomol (Plymouth Meeting, PA). For in vitro kinase assays, histone H1 was purchased from Upstate Biotechnology (Lake Placid, NY) and Redivue [γ-^{32}P]ATP was purchased from Amersham Pharmacia Biotech (Baie d'Urfe, Quebec, Canada).

Cells

The murine B lymphoma cell lines WEHI-231 and M12.4.1 were grown in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 1 mM sodium pyruvate, and 50 μM 2-ME (culture medium). M12.4.1 cells expressing the superrepressor form of IκBα (64) under the control of an inducible promoter were maintained in culture medium supplemented with 0.4 mg/ml G418 (Life Technologies, Burlington, Ontario, Canada). To induce the expression of the IκBα superrepressor in these cells, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 200 μM 24 h before stimulating the cells. Small resting B cells were isolated from the spleens of BALB/c mice by Percoll (Amersham Pharmacia Biotech) density centrifugation after killing T cells using a mixture of guinea pig complement plus mAbs to murine Thy1, CD4, and CD8 (65). FACS analysis showed that the resulting population of cells isolated from the interface of the 60 and 75% Percoll layers was >95% IgM$^+$.

Cell stimulation

The WEHI-231 and M12.4.1 cells were resuspended in culture medium at 2×10^6/ml for time points of 4 h or less and at 5×10^5/ml for longer time points. Small resting B cells from mouse spleen were resuspended to 5×10^6 cells/ml in culture medium. Cells were stimulated with 5 μg/ml of the 1C10 anti-CD40 mAb, 12 μg/ml goat anti-mouse IgM Abs, or 5 μg/ml LPS. Where indicated, cycloheximide (2 μg/ml final concentration) was added to the cells at the same time as the stimuli while the NF-κB inhibitor BAY11-7082 was added to the cells 1–2 h before stimulation.

Preparation of total RNA

After stimulation, the cells were pelleted and total RNA was isolated using TRIzol (Life Technologies). The RNA was dissolved in H_2O and concentrations were determined by measuring A_{260}. When the RNA was to be used for gene array analysis, it was incubated with 5 U of DNase I (Ambion, Austin, TX) for 30 min at 37°C to remove any contaminating genomic DNA. This reaction was stopped by adding one-tenth volume of 10× termination mix (0.1 M EDTA (pH 8), 1 mg/ml glycogen) and the RNA was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1). After ethanol precipitation, the RNA was washed once with 80% ethanol and then dissolved in deionized H_2O with RNase inhibitors (Ambion).

Gene expression profiling using Atlas cDNA arrays

Matched pairs of Atlas mouse cDNA expression array I membranes were purchased from Clontech Laboratories (catalog no. 7741-1). These membranes are spotted in duplicate with 588 selected partial mouse cDNAs. ^{32}P-labeled first-strand cDNA probes were generated by incubating 2 μg of total RNA from each cell population with Moloney murine leukemia virus reverse transcriptase and pooled primers specific for the 588 genes. Unincorporated nucleotides were removed using ChromaSpin columns (Clontech Laboratories). After prehybridizing the membranes for 1 h with ExpressHyb (Clontech Laboratories) containing 0.5 mg/ml sheared salmon testes DNA (Ambion), the membranes were incubated in hybridization bottles with 10 ml of ExpressHyb containing 5×10^6 cpm of denatured ^{32}P-labeled cDNA probe. Hybridization was performed overnight at 68°C in a hybridization oven, rotating the bottles at 5 rpm. The membranes were then washed extensively and exposed to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA) for 3–5 days at 4°C, and the resulting hybridization signals were measured using a PSI PhosphorImager (Molecular Dynamics). Atlas Image 1.0 (Clontech Laboratories) software was used to quantify the hybridization signals. The intensities for each spot were corrected for background levels and normalized for differences in probe labeling by using the average values for multiple housekeeping genes (GAPDH, α-actin, ubiquitin, calcium-binding protein, ribosomal protein S29) whose expression did not change significantly upon cell stimulation.

Preparation of probes for Northern blotting

An IMAGE clone containing 453 bp of the 3′ noncoding sequence of the murine *pim-1* gene (GenBank accession no. AA764204) cloned into the pT7T3 vector was purchased from Genome Systems (St. Louis, MO). To generate a probe for Northern blotting, this portion of the *pim-1* gene was amplified by PCR using standard T7 and T3 primers. The resulting PCR product was purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA). The GAPDH probe for Northern blotting was generated by in vitro transcription. Briefly, a pSP72 vector (Promega, Madison, WI) containing 780 bp of the 5′ noncoding sequence plus the first 250 codons of the human GAPDH gene was linearized with *Xba*I and then incubated with T7 RNA polymerase (Ambion). Purified DNA or RNA probes (0.5 μg) were labeled using the BrightStar psoralen-biotin nonisotopic labeling kit (Ambion) according to the manufacturer's instructions.

Northern blotting

Northern blots were conducted using the Northern Max Northern Blotting kit (Ambion). Briefly, 20 μg of total RNA was loaded in each well of a 1% agarose formaldehyde gel and subjected to electrophoresis at 70 V for 2 h. RNA Millennium Markers (Ambion) were loaded as RNA size markers. The gel was photographed with UV light on a trans-illuminator to ensure equal loading and to determine the migration of the RNA size markers. The RNA was then electrophoretically transferred to a BrightStar-Plus positively charged nylon membrane (Ambion) for 1 h at 100 V using a Bio-Rad trans-blot cell (Bio-Rad, Hercules, CA) and 0.5× TBE (45 mM Tris-borate (pH 8), 1 mM EDTA) as the transfer buffer. The membrane was baked at 80°C for 15 min to cross-link the RNA to the filter. Prehybridization, as well as hybridization with the *pim-1* DNA probe, was performed at 42°C according to the manufacturer's instructions (Ambion). When the GAPDH riboprobe was used, both the prehybridization and hybridization steps were done at 68°C. The membranes were then washed and the binding of the probes to the membranes was visualized using the BrightStar BioDetect nonisotopic detection kit (Ambion). The membranes were exposed to x-ray film and the hybridization signals were quantified by scanning the films and analyzing the resulting TIFF files using ImageQuant software (Molecular Dynamics). The relative intensity of each band was determined by normalizing to the intensities of the corresponding GAPDH bands.

Preparation of cell extracts and immunoblotting

After stimulation, the cells were washed once with PBS and then resuspended in 100 μl of ice-cold buffer A (10 mM HEPES-KOH (pH 7.8), 10 mM KCl, 1.5 mM $MgCl_2$, 20% glycerol, 25 mM NaF, 0.5 mM Na_3VO_4, 0.5 mM DTT, 2 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF). After 15 min on ice, 5 μl of 10% Nonidet P-40 was added. The samples were then centrifuged at 7,000 rpm for 4 min at 4°C and the supernatants were collected as cytoplasmic extracts. In some experiments, the pellets were resuspended in 60 μl of buffer B (20 mM HEPES-KOH (pH 7.8), 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 25 mM NaF, 0.5 mM Na_3VO_4, 0.5 mM DTT, 2 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF) to extract nuclear proteins. After 20 min on ice, the samples were centrifuged at 14,000 rpm for 4 min at 4°C and the supernatants were collected as nuclear extracts. Protein concentrations were determined using the BCA assay (Pierce, Rockford, IL). Sixty micrograms of either cytoplasmic extracts ($\sim 1 \times 10^6$ cell equivalents for cell lines; 5×10^6 cell equivalents for splenic B cells) or nuclear extracts ($\sim 4 \times 10^6$ cell equivalents for cell lines) were separated on 12% SDS-PAGE gels and transferred to nitrocellulose filters. The filters were blocked for 30 min at room temperature with PBS/0.05% Tween 20 (PBST) containing 5% skim milk powder. After washing with PBST, the filters were incubated with primary Ab (diluted in PBST) overnight at 4°C or 1 h at room temperature. The filters were then washed with PBST and incubated with HRP-conjugated secondary Ab in PBST containing 5% skim milk powder for 30 min at room temperature. After washing, the immunoreactive bands were visualized using ECL detection (Amersham Pharmacia Biotech) and quantitated either by densitometry using an Alpha Innotech gel documentation system (Canberra Packard, Mississauga, Ontario, Canada) or by scanning the films and using ImageQuant software. Where indicated, the filters were reprobed after previously bound Abs were eluted by washing the filter in 10 mM Tris-HCl (pH 2)/150 mM NaCl for 20 min at room temperature.

Pim-1 in vitro kinase assays

Cytoplasmic extracts (100 μg) were diluted in 0.5 ml of buffer C (50 mM Tris-HCl (pH 7.8), 250 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 25 mM NaF, 0.5 mM Na_3VO_4, 0.5 mM DTT, 2 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF). The samples were then precleared for 1 h at 4°C with 10 μl of preimmune rabbit serum and 10 μl of protein A-Sepharose. After spinning out the beads, the samples were incubated for 1 h at 4°C with 5 μl of anti-Pim-1 Abs or preimmune serum. Immune complexes were collected by adding 10 μl of protein A-Sepharose and incubating for an additional hour. The beads were then washed three times with 1 ml of buffer C and twice with 1 ml of Pim-1 kinase buffer (25 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 0.5 mM DTT) (66) before being resuspended in 30 μl of Pim-1 kinase buffer containing 10 μM ATP, 10 μCi of [γ-^{32}P]ATP, and 25 μg of histone H1. A purified GST-Pim-1 fusion protein (43) was used as a positive control. After 30 min at 25°C, the reactions were stopped by adding 15 μl of 3× SDS-PAGE sample buffer. Twenty microliters of each sample was separated on a 15% SDS-PAGE gel and transferred to nitrocellulose filters. The filters were dried and exposed to x-ray film or analyzed using a PhosphorImager (Molecular Dynamics).

Results

pim-1 *mRNA levels increase after CD40 engagement*

To screen for genes whose expression is regulated by CD40 signaling in the WEHI-231 murine B cell line, we used Clontech Atlas cDNA arrays, a gene expression profiling system that we have used previously to identify genes that are regulated during macrophage activation (67, 68). These Atlas cDNA array membranes are spotted in duplicate with 588 different cDNAs, many of which encode proteins involved in apoptosis, proliferation, differentiation, and cell-cell communication. RNA was prepared from unstimulated WEHI-231 cells and from cells cultured for 4 h with the 1C10 anti-CD40 mAb. Stimulating WEHI-231 cells with this mAb initiates CD40-dependent signaling events including the activation of NF-κB and the activation of the JNK and p38 MAP kinases (25, 69). Moreover, culturing WEHI-231 cells with this mAb also prevents anti-IgM-induced growth arrest (60). The RNA isolated from unstimulated or 1C10-stimulated WEHI-231 cells was used to generate cDNA probes by RT-PCR for the 588 genes immobilized on the Atlas gene array membranes. Probing the Atlas cDNA array membranes with these pooled cDNA probes revealed a number of potential CD40-regulated genes including *pim-1* (GenBank accession no. M13945). A representative portion of the cDNA array membranes depicting the anti-CD40-stimulated increase in *pim-1* hybridization is shown in Fig. 1*A*. The hybridization signals for many other genes, including a nearby gene on the membrane, the Ets-related protooncogene *fli-1*, did not change upon CD40 engagement.

Northern blots were performed to confirm that CD40 signaling increased *pim-1* mRNA levels. For these experiments we used a probe corresponding to the 3′ untranslated region of the *pim-1* gene so that we would detect only full-length mRNA transcripts. The hematopoietic form of murine *pim-1* mRNA has been reported to be 2.8 kb (70), and Fig. 1*B* shows that stimulating WEHI-231 cells with the 1C10 anti-CD40 mAb increased the levels of the 2.8-kb *pim-1* mRNA. The anti-CD40-induced increase in *pim-1* mRNA levels could be detected within 30 min, peaked at 30–60 min, and persisted for at least 4 h. At 30–60 min after adding 1C10 to the cells, *pim-1* mRNA levels were ~4.5 times the basal level. The rapid increase in *pim-1* mRNA levels after CD40 engagement suggests that *pim-1* is an immediate early gene whose induction is mediated by pre-existing proteins. Consistent with this idea, stimulating WEHI-231 cells with anti-CD40 Abs caused a similar increase in *pim-1* mRNA levels in both the presence and absence of the protein synthesis inhibitor cycloheximide (Fig. 1*C*).

Pim-1 protein levels and kinase activity are increased after CD40 signaling

To determine whether the CD40-induced increase in *pim-1* mRNA levels was translated into an increase in Pim-1 protein levels, we performed immunoblots using anti-Pim-1 Abs. The murine *pim-1* gene encodes two forms of Pim-1, a 33-kDa protein (p33 Pim-1) and a 44-kDa protein (p44 Pim-1). p33 Pim-1 consists almost entirely of a kinase domain. p44 Pim-1 is encoded by the same gene as p33 Pim-1, but translation is initiated at an upstream CUG codon (42). This results in p44 Pim-1 having a unique 11-kDa N-terminal extension that is followed by the sequence of the p33 Pim-1 protein. These two isoforms of murine Pim-1 have been reported to have comparable kinase activity and both can prevent apoptosis (53). Fig. 2*A* shows that CD40 engagement caused a significant increase in the levels of both the 33- and 44-kDa forms of Pim-1 in cytoplasmic extracts from WEHI-231 cells. CD40 signaling also increased Pim-1 levels in small resting B cells from mouse spleen (Fig. 2*B*) and in another murine B cell line, M12.4.1

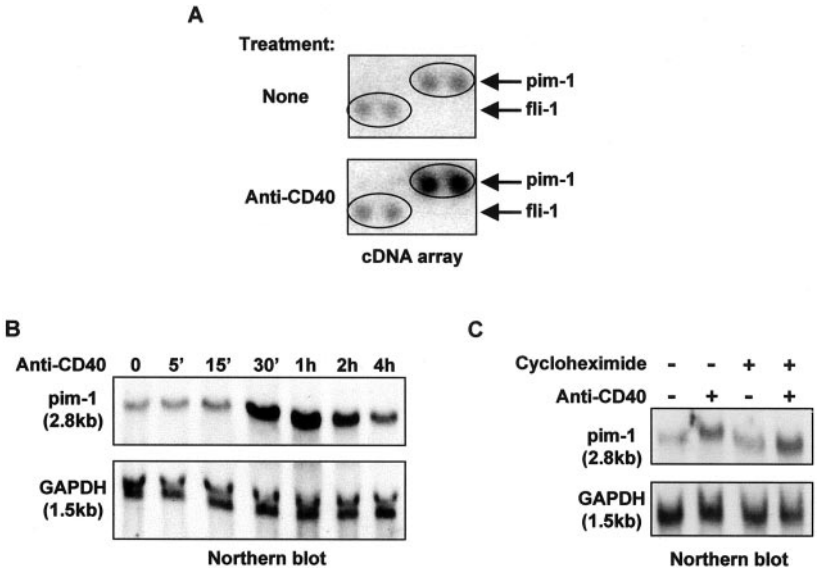

FIGURE 1. CD40 signaling increases the levels of *pim-1* mRNA in WEHI-231 cells. *A*, WEHI-231 cells were cultured with or without the 1C10 anti-CD40 mAb (3 μg/ml) for 4 h. RNA isolated from these cells was used to generate pools of ^{32}P-labeled cDNA probes specific for the 588 different cDNAs immobilized on the Atlas cDNA array membranes. These pools of cDNA probes were incubated with the membranes, and the hybridization signal for each of the 588 immobilized cDNAs was visualized and quantitated using a PhosphorImager. A portion of the membranes that includes the duplicate spots for the *pim-1* cDNA and the nearby *fli-1* cDNA is shown. Note that there was no detectable hybridization of the probes to the immobilized cDNAs spotted in the *upper left corner* (*elk-1*) or the *lower right corner* (c-*fms*) of this portion of the membranes. Quantitation revealed that the *pim-1* hybridization signal increased by 1.5- to 1.8-fold after CD40 engagement ($n = 3$; hybridization signals for the unstimulated and anti-CD40-stimulated samples were normalized using five housekeeping genes whose expression did not change significantly upon CD40 engagement). *B*, WEHI-231 cells were cultured with the 1C10 anti-CD40 mAb (5 μg/ml) for the indicated times. Total RNA was isolated and analyzed by Northern blotting with a ^{32}P-labeled probe for *pim-1* mRNA (2.8 kb). To control for loading, the filter was reprobed for GAPDH mRNA (1.5 kb). *pim-1* hybridization signals were quantitated by densitometry and normalized to the corresponding GAPDH values for each sample. This quantitation showed that CD40 signaling increased *pim-1* mRNA levels by 4- to 4.5-fold at the 30- and 60-min time points. Note also that the CD40-induced increase in *pim-1* mRNA levels at the 4-h time point, as determined by Northern blotting, was similar in magnitude (~1.8-fold) to the increase in *pim-1* hybridization seen using the cDNA array membranes (Fig. 1*A*). *C*, WEHI-231 cells were cultured for 4 h with or without the 1C10 anti-CD40 mAb (5 μg/ml) in the presence or absence of 2 μg/ml cycloheximide. *pim-1* mRNA levels were determined by Northern blotting. As a loading control, the filter was reprobed for GAPDH mRNA (1.5 kb). For each panel, similar results were obtained in at least two independent experiments.

(Fig. 2*C*). In contrast to WEHI-231, which is an IgM$^+$ immature B cell line, M12.4.1 is an IgG$^+$ mature B cell line. The CD40-induced increases in Pim-1 protein levels were maximal at 2–4 h and persisted for at least 24 h. In other experiments (see Figs. 4*A* and 5*B*), we found that increases in Pim-1 levels could be seen within 30–60 min of adding the 1C10 anti-CD40 mAb to WEHI-231 cells or to M12.4.1 cells. Thus, up-regulation of Pim-1 protein levels is a rapid response to CD40 engagement.

Because Pim-1 must be a functional kinase to promote cell proliferation and survival (53, 71), we asked whether CD40 signaling resulted in an increase in Pim-1 kinase activity. To test this, we immunoprecipitated Pim-1 from cytoplasmic extracts of WEHI-231 cells and assayed its ability to phosphorylate histone H1 in vitro. We found that stimulating WEHI-231 cells with the 1C10 anti-CD40 mAb caused a 3-fold increase in total Pim-1 kinase activity at 2 h and a 2.5-fold increase at 4 h (Fig. 3). These CD40-induced increases in total Pim-1 kinase activity were similar in magnitude to the increases in Pim-1 protein levels seen in this and other experiments (see also Fig. 6*B*). This indicates that the increase in total Pim-1 kinase activity is due mainly to increased levels of the Pim-1 protein as opposed to an increase in the specific activity of Pim-1. In any case, this CD40-induced increase in total Pim-1 kinase activity could lead to increased phosphorylation of Pim-1 substrates in B cells.

Up-regulation of Pim-1 by CD40 involves the NF-κB pathway

Thus far we have shown that CD40 signaling increases the levels of *pim-1* mRNA as well as Pim-1 protein. Next, we wished to understand how CD40 regulates the expression of Pim-1. CD40 activates multiple signaling pathways including those leading to the activation of NF-κB, PI3K, ERK, JNK, and p38 MAP kinase. To investigate the role of these signaling pathways in linking CD40 engagement to the up-regulation of Pim-1, we started by using a variety of chemical inhibitors. Preliminary results showed that specific inhibitors of the PI3K, ERK, and p38 pathways (LY294002, PD90859, and SB203580, respectively) did not have a significant effect on the ability of CD40 to increase Pim-1 protein levels (data not shown). Therefore, we focused on the NF-κB pathway, which plays a key role in the ability of CD40 to promote the survival of WEHI-231 cells (34, 72–74) and the proliferation of normal murine B cells (75, 76). To determine whether CD40 regulates Pim-1 levels via NF-κB, we used two different approaches to inhibit the activation of NF-κB.

The first way in which we inhibited NF-κB activation was to pretreat cells with BAY11-7082 (62, 63), a cell-permeable chemical inhibitor of the kinases that phosphorylate the IκB proteins. IκB proteins normally inhibit NF-κB activation by binding NF-κB in the cytoplasm and preventing it from translocating into the nucleus. Receptor-induced activation of the IκB kinase complex results in the phosphorylation of IκB, marking it for ubiquitination and subsequent proteasome-mediated degradation. Once IκB is degraded, NF-κB can translocate into the nucleus and promote transcription. By preventing the phosphorylation and degradation of IκB, BAY11-7082 can prevent the activation of NF-κB.

We found that BAY11-7082 was an effective inhibitor of CD40-induced IκBα phosphorylation (Fig. 4*A*) in WEHI-231 cells. As

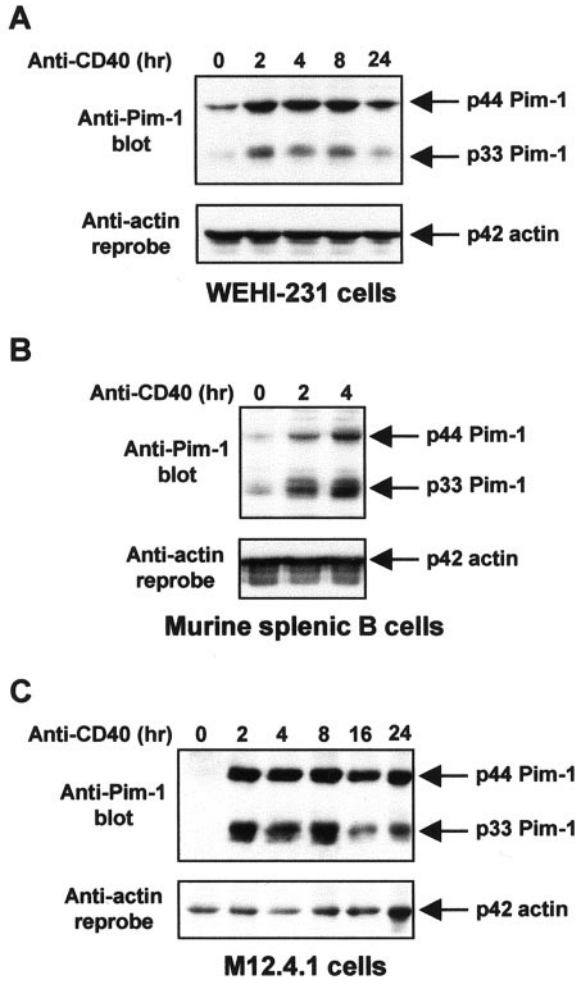

FIGURE 2. CD40 signaling increases Pim-1 protein levels. WEHI-231 cells (*A*), small resting B cells from mouse spleen (*B*), and M12.4.1 cells (*C*) were cultured with the 1C10 anti-CD40 mAb (5 μg/ml) for the indicated times. Cytoplasmic extracts were separated by SDS-PAGE and then analyzed by immunoblotting with an Ab that recognizes both the 44- and 33-kDa forms of Pim-1. As a loading control, the filters were stripped and reprobed with anti-actin Abs. For each cell type, similar results were obtained in at least two independent experiments.

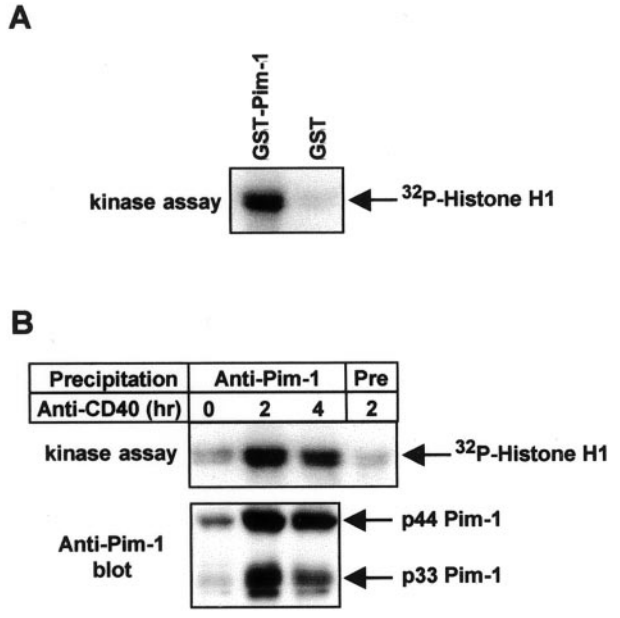

FIGURE 3. CD40 signaling increases Pim-1 kinase activity in WEHI-231 cells. *A*, Histone H1 is a substrate for a recombinant GST-Pim-1 fusion protein but is not phosphorylated by GST. *B*, WEHI-231 cells were cultured with the 1C10 anti-CD40 mAb (5 μg/ml) for the indicated times. *Upper panel*, Pim-1 was immunoprecipitated from cytoplasmic extracts and in vitro kinase assays were performed using histone H1 as the substrate. As a control, extracts from anti-CD40-stimulated cells were precipitated with preimmune serum (Pre) instead of anti-Pim-1 Abs. *Lower panel*, separate aliquots of the cytoplasmic extracts that were used for the in vitro kinase assays were analyzed for Pim-1 protein levels by immunoblotting. Similar results were obtained in two independent experiments.

shown in Fig. 4*A* and in our previous work (69), CD40 engagement causes a rapid initial phosphorylation of IκBα that is maximal at 5 min. This phosphorylated IκBα is then rapidly degraded such that very little IκBα is present at 15 min (59). IκBα is then resynthesized, and this is accompanied by a second, sustained wave of IκBα phosphorylation which is evident at 30 min to 2 h after the addition of the anti-CD40 mAb to the WEHI-231 cells (Fig. 4*A*). In contrast, when the cells were pretreated with BAY11-7082 for 1 h, the initial CD40-induced IκBα phosphorylation was substantially reduced and the second wave of IκBα phosphorylation that is normally seen at 30 min to 2 h did not occur. Note that while BAY11-7082 blocks IκBα phosphorylation, it does not inhibit the kinases involved in the activation of the ERK, JNK, or p38 MAP kinase (62). Thus, BAY11-7082 allows one to distinguish CD40-induced responses that are dependent on NF-κB activation from responses that are dependent on the activation of MAP kinases.

Having shown that BAY11-7082 can effectively block IκBα phosphorylation (Fig. 4*A*) and degradation (data not shown) in WEHI-231 cells, we asked whether this impaired the ability of CD40 to induce Pim-1 expression. We found that the initial CD40-induced up-regulation of p44 Pim-1 and p33 Pim-1 that occurs at 30 min to 2 h after the addition of the 1C10 mAb to the cells was almost completely blocked when the cells were pretreated with 60 μM BAY11-7082 (Fig. 4*A*). Similar results were observed in the mature B cell line M12.4.1. Pretreating these cells with 60 μM BAY11-7082 completely blocked the CD40-induced up-regulation of Pim-1 that is normally seen at 2 h (Fig. 4*B*). Note that at lower cell density (4×10^5/ml instead of 2×10^6/ml), 20 μM BAY11-7082 could fully inhibit CD40-induced Pim-1 expression in both WEHI-231 cells and M12.4.1 cells (data not shown). These results indicate that the initial up-regulation of Pim-1 protein levels by CD40 is largely dependent on the activation of NF-κB.

To strengthen our conclusion that CD40 induces Pim-1 expression via NF-κB, we used a second, mechanistically distinct approach to inhibit NF-κB activation. We made use of M12.4.1 cells that express a superrepressor form of IκBα under the control of an IPTG-inducible promoter. This IκBα superrepressor has serine to alanine mutations at residues 32 and 36 such that it cannot be phosphorylated by the IκB kinase complex. Therefore, it is a stable form of IκBα that upon receptor signaling is not degraded but instead continues to restrain NF-κB in the cytoplasm. This IκBα superrepressor is an effective inhibitor of NF-κB activation (77) and has been used previously to inhibit CD40-induced NF-κB activation in M12.4.1 cells (64). We confirmed this by showing that inducing the expression of the IκBα superrepressor in M12.4.1 cells significantly reduced CD40-stimulated nuclear translocation of the p65 RelA subunit of NF-κB (Fig. 5*A*). In cells expressing the IκBα superrepressor, the amount of RelA in the nucleus after CD40 engagement was ~70% less than in cells in which the IκBα superrepressor was not induced. Under these conditions in which NF-κB activation was substantially reduced, we found that the

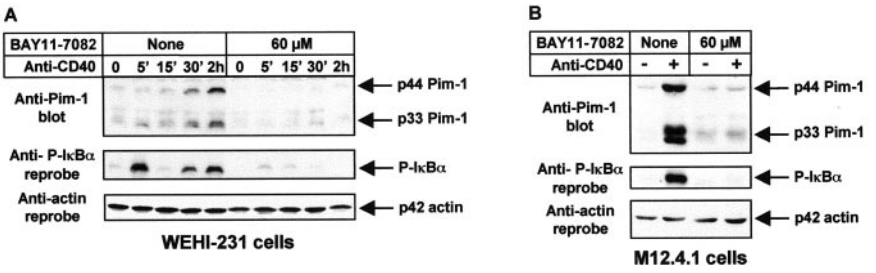

FIGURE 4. CD40-induced up-regulation of Pim-1 is blocked by BAY11-7082, an inhibitor of IκBα phosphorylation. *A*, WEHI-231 cells were cultured with or without 60 μM BAY11-7082 for 1 h before being stimulated with the 1C10 anti-CD40 mAb (5 μg/ml) for the indicated times. Cytoplasmic extracts were separated by SDS-PAGE and transferred to nitrocellulose. The filters were probed sequentially with Abs to Pim-1, phosphorylated IκBα (P-IκBα), and actin. *B*, M12.4.1 cells were cultured with or without 60 μM BAY11-7082 for 2 h before being stimulated with the 1C10 anti-CD40 mAb for 2 h. Cell extracts were separated by SDS-PAGE and immunoblotted sequentially with Abs to Pim-1, phosphorylated IκBα (P-IκBα), and actin. For each panel, similar results were obtained in two independent experiments.

ability of the 1C10 anti-CD40 mAb to induce the expression of Pim-1 was inhibited by >70% (Fig. 5*B*). The residual up-regulation of Pim-1 that occurred when the IκBα superrepressor was expressed could be due to the incomplete inhibition of NF-κB activation or to other CD40 signaling pathways. In either case, these results, together with those in Fig. 4, argue that CD40 regulates Pim-1 protein levels primarily via the activation of NF-κB.

Up-regulation and/or maintenance of Pim-1 levels correlates with the survival of WEHI-231 cells

WEHI-231 cells have been used as a model for Ag-induced clonal deletion because treating these cells with anti-IgM Abs results in growth arrest in the G_1 phase (78) of the cell cycle followed by apoptosis (79, 80). This anti-IgM-induced growth arrest is nearly complete after 18–24 h, and by 48 h most of the cells are dead (81). Moreover, by 18 h after the addition of anti-IgM, >90% of the cells are irreversibly committed to growth arrest (81). In contrast, when WEHI-231 cells are cultured with either anti-CD40 Abs or soluble CD40L in addition to anti-IgM Abs, CD40 initiates survival signals that prevent the anti-IgM-induced growth arrest and apoptosis (4, 40). Because Pim-1 expression is associated with cell survival and proliferation, we hypothesized that anti-IgM treatment of WEHI-231 cells might cause growth arrest by suppressing Pim-1 expression while CD40-induced up-regulation of Pim-1 might prevent the anti-IgM-induced growth arrest.

To explore this hypothesis, we added either anti-IgM Abs, the 1C10 anti-CD40 mAb, or both of these Abs to WEHI-231 cells and then cultured the cells for 2–24 h before analyzing the levels of p44 Pim-1 and p33 Pim-1 by immunoblotting (Fig. 6). We found

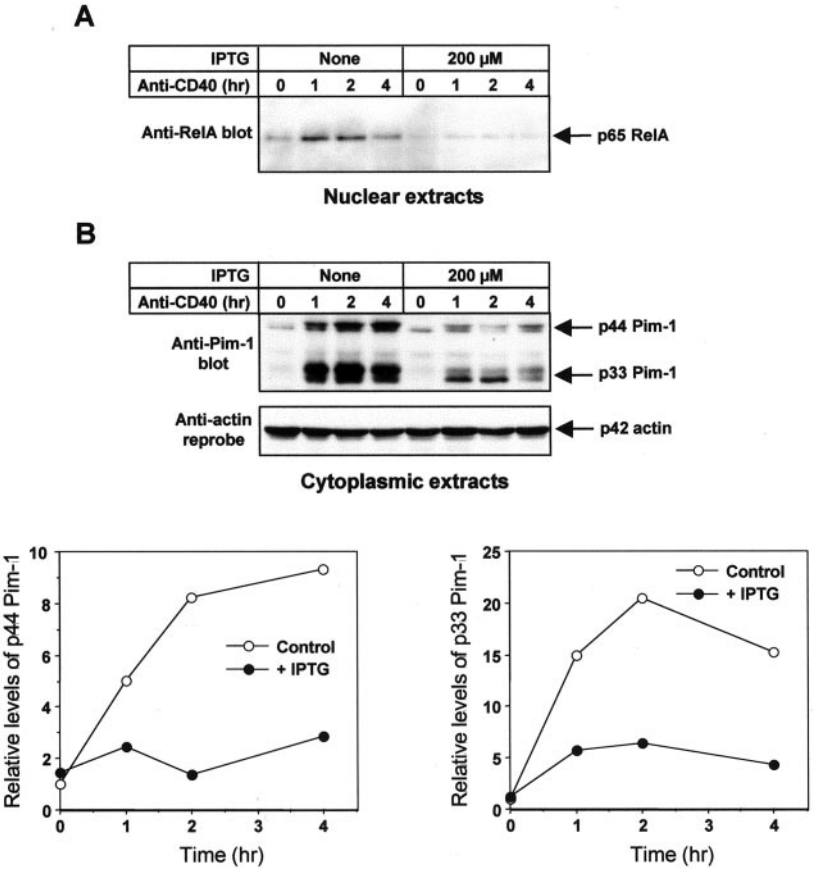

FIGURE 5. CD40-induced up-regulation of Pim-1 is blocked by expression of the IκBα superrepressor. M12.4.1 cells that express the IκBα superrepressor under the control of an IPTG-inducible promoter were cultured with or without 200 μM IPTG for 24 h. The cells were then stimulated with the 1C10 anti-CD40 mAb (5 μg/ml) for the indicated times. *A*, Nuclear extracts were separated by SDS-PAGE and immunoblotted with Abs to the p65 RelA subunit of NF-κB. *B*, Cytoplasmic extracts from the same samples as in *A* were separated by SDS-PAGE and transferred to nitrocellulose. *Upper panel*, the filters were probed sequentially with Abs to Pim-1 and actin. *Lower panels*, the levels of p44 Pim-1 and p33 Pim-1 in this experiment were determined by densitometry and normalized to the amount of actin (loading control) for each sample. The levels of p44 Pim-1 and p33 Pim-1 in unstimulated cells that had been cultured in the absence of IPTG were each assigned a value of 1. Similar results were obtained in two independent experiments.

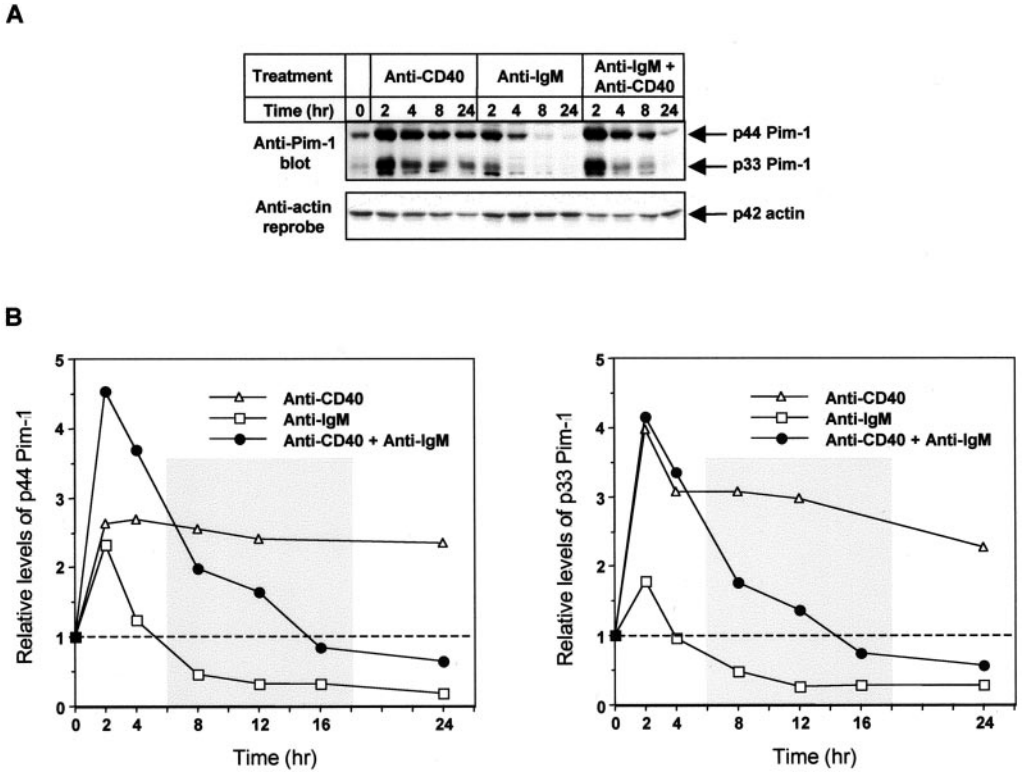

FIGURE 6. CD40-induced up-regulation/maintenance of Pim-1 levels correlates with WEHI-231 survival. *A*, WEHI-231 cells were stimulated with 1C10 anti-CD40 mAb (5 μg/ml), anti-IgM Abs (12 μg/ml), or with both Abs, for the indicated times. Pim-1 expression was analyzed by immunoblotting cytoplasmic extracts with anti-Pim-1 Abs. The filters were then stripped and reprobed with anti-actin Abs. A representative experiment is shown. *B*, The levels of p44 Pim-1 and p33 Pim-1 in five independent time course experiments were determined by densitometry and normalized to the amount of actin in the same sample. The basal levels of p44 Pim-1 and p33 Pim-1 in unstimulated cells were each assigned a value of 1 and are indicated by the dashed lines. Each data point represents the mean value of data points from two to five experiments. The SEMs were $<$20% of the mean value. The shaded box indicates the time period during which WEHI-231 cells cultured with anti-IgM alone would irreversibly commit to growth arrest (81).

that anti-IgM treatment caused an initial transient increase in the levels of both p44 Pim-1 and p33 Pim-1 that could be detected as early 30 min (data not shown), and which peaked at 2 h. After this initial increase, Pim-1 levels in the anti-IgM-treated cells decreased, declining to below basal levels by 8 h and declining further to barely detectable levels by 24 h. By 36 h, the majority of the cells were dead. Thus, anti-IgM-induced growth arrest in WEHI-231 cells was accompanied by down-regulation of Pim-1 levels. In contrast to anti-IgM, CD40 engagement by itself caused a sustained increase in Pim-1 levels. We then examined what happened when the cells were cultured with anti-IgM plus anti-CD40, a situation in which CD40 signaling prevents anti-IgM-induced growth arrest and allows the cells to survive and proliferate for at least 48 h. Under these conditions, we found that there was a large initial increase in Pim-1 levels which again peaked at 2 h before beginning to decline. However, Pim-1 levels in the cells treated with anti-IgM plus anti-CD40 remained above basal levels for at least 12 h and, at both 16 and 24 h, Pim-1 levels in these cells were significantly higher than in cells treated with anti-IgM only. Thus, at early times (2–12 h) CD40 signaling can up-regulate Pim-1 levels even in the presence of anti-IgM, while at later times (16–24 h) it opposes the dramatic decrease in Pim-1 levels that is caused by anti-IgM. In particular, during the critical time period from 6 to 18 h after anti-IgM addition, when cells treated with anti-IgM alone would irreversibly commit to growth arrest (81), coculture with anti-CD40 Abs significantly increases Pim-1 levels compared with those in cells cultured with anti-IgM alone. These correlative data suggest that the ability of CD40 to maintain Pim-1 levels above a certain threshold could contribute to the prevention of anti-IgM-induced growth arrest.

To extend this correlation between Pim-1 expression and the survival of WEHI-231 cells, we asked whether the ability of bacterial LPS to prevent anti-IgM-induced growth arrest in these cells also correlated with up-regulation or maintenance of Pim-1 levels. LPS is a potent mitogen for murine B cells and culturing WEHI-231 cells with $\geq$ 1 μg/ml LPS can completely protect these cells from anti-IgM-induced growth arrest (82). Moreover, there is considerable overlap between LPS-induced signaling and CD40 signaling. Both the Toll-like receptor 4-containing LPS receptor complex (83) and CD40 interact with TRAF proteins and both activate NF-κB as well as ERK, JNK, and p38 MAP kinase. We found that LPS by itself increased Pim-1 levels in both murine splenic B cells (Fig. 7*A*) and WEHI-231 cells (Fig. 7, *B–D*). In WEHI-231 cells, the LPS-induced increase in Pim-1 levels was sustained for at least 24 h (Fig. 7, *B–D*). When WEHI-231 cells were cultured with both anti-IgM and LPS, a situation in which LPS prevents anti-IgM-induced growth arrest, there was a large initial increase in Pim-1 levels at 2 h which was followed by a slower decline in Pim-1 levels, similar to what was observed when these cells were cultured with anti-IgM plus anti-CD40. Significantly, LPS, like anti-CD40, prevented the dramatic down-regulation of Pim-1 that is caused by anti-IgM in WEHI-231 cells. While anti-IgM treatment reduced Pim-1 expression to nearly undetectable levels, Pim-1 levels in WEHI-231 cells cultured with anti-IgM plus LPS remained at or above basal levels for at least 24 h. Thus, the ability of both anti-CD40 and LPS to prevent the anti-IgM-induced growth arrest

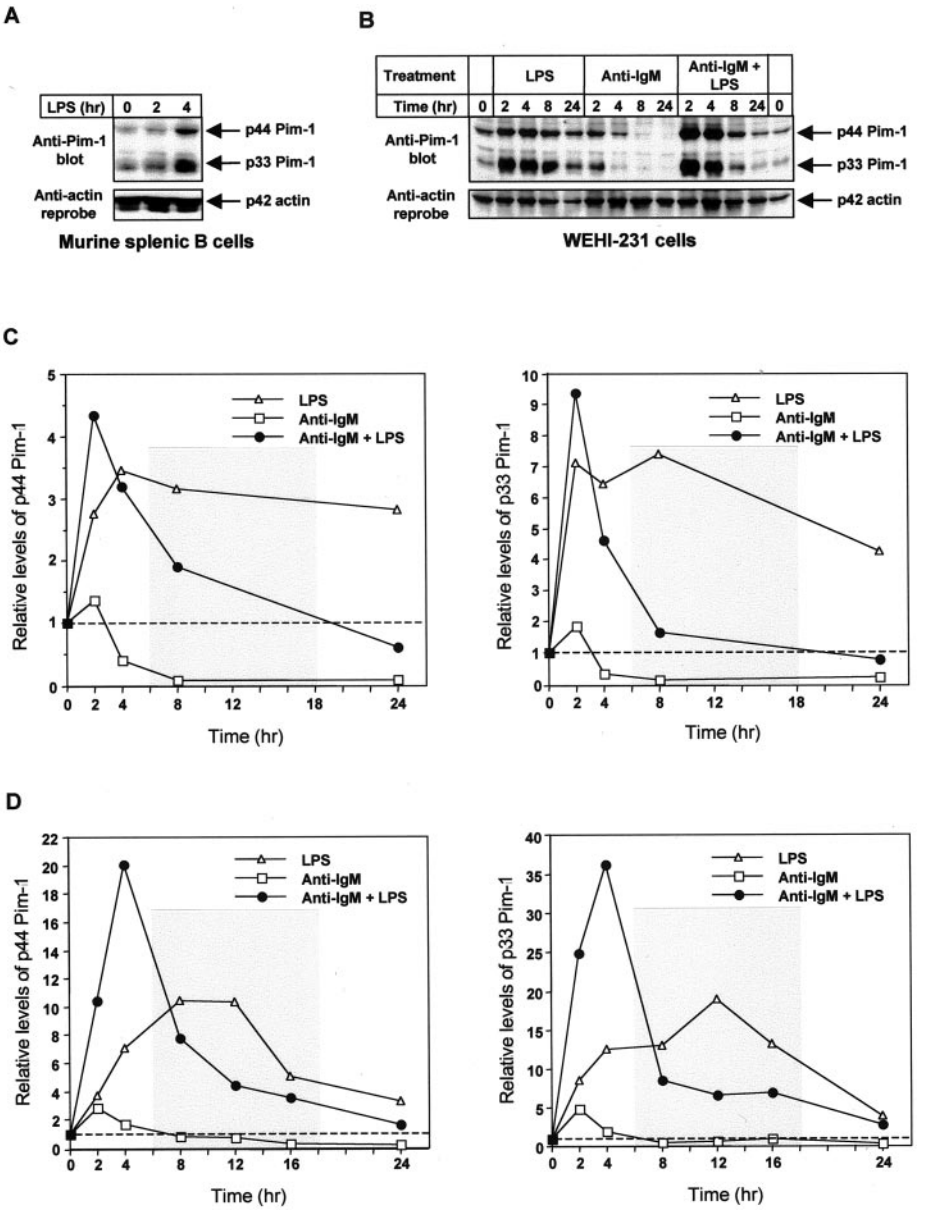

FIGURE 7. LPS-induced up-regulation/maintenance of Pim-1 levels. *A*, Murine splenic B cells were stimulated with 5 μg/ml LPS for the indicated times. Pim-1 expression was analyzed by immunoblotting cytoplasmic extracts with anti-Pim-1 Abs. Similar results were obtained in two independent experiments. *B–D*, WEHI-231 cells were stimulated with 5 μg/ml LPS, 12 μg/ml anti-IgM Abs, or both for the indicated times. Pim-1 expression was analyzed by immunoblotting. The filters were then stripped and reprobed with anti-actin Abs. A representative blot is shown in *B*. *C*, The quantitation of the blot in *B*. *D*, The quantitation of another independent experiment. The levels of p44 Pim-1 and p33 Pim-1 were determined by densitometry and normalized to the amount of actin for each sample. The basal levels of p44 Pim-1 and p33 Pim-1 in unstimulated cells (average of two independent cultures) were each assigned a value of 1 and are indicated by the dashed lines. The shaded box indicates the time period during which WEHI-231 cells cultured with anti-IgM alone would irreversibly commit to growth arrest (81).

of WEHI-231 cells correlates with their ability to oppose the anti-IgM-induced down-regulation of Pim-1.

Discussion

Pim-1 is a serine/threonine kinase whose expression can promote cell survival and proliferation. In this report, we have made a number of novel findings concerning Pim-1 in B cells. First, we showed that the *pim-1* gene is a target of CD40 signaling and that CD40 engagement results in a rapid increase in Pim-1 protein levels and total Pim-1 kinase activity. Second, we showed that the ability of CD40 to increase the Pim-1 levels depends on the activation of NF-κB. Third, we showed that both LPS stimulation and BCR engagement also regulate Pim-1 levels in B cells. Fourth, we showed that signals emanating from CD40, the LPS receptor complex, and the BCR are integrated at the level of Pim-1 in the WEHI-231 B lymphoma cell line. Finally, we showed that up-regulation and/or maintenance of Pim-1 levels correlates with the survival of WEHI-231 cells.

Although many cytokines and mitogens increase Pim-1 levels in hematopoietic cells, our finding that CD40 regulates Pim-1 protein levels via NF-κB is the first report linking NF-κB activation to *pim-1* expression. This suggests that other receptors that activate NF-κB could also up-regulate Pim-1 levels, at least in B cells. These receptors could include other members of the TNFR family besides CD40, as well as the LPS receptor complex and the BCR. Indeed, we showed that LPS treatment causes sustained up-regulation of Pim-1 levels in B cells while BCR signaling causes a transient increase in Pim-1 levels. Moreover, this BCR-induced increase in Pim-1 levels is blocked by the NF-κB inhibitor BAY11-7082 (data not shown).

Because the promoter for the human *pim-1* gene contains a potential NF-κB binding site (84), the simplest model for the regulation of Pim-1 by CD40 is that CD40-induced NF-κB activation increases the transcription of the *pim-1* gene and that this is translated into an increase in Pim-1 protein levels. Consistent with this idea, our preliminary data show that CD40 engagement can cause a 1.8- to 2-fold increase in the transcription of a luciferase reporter gene (data not shown) that is under the control of the 780-bp proximal *pim-1* promoter (84). However, because *pim-1* mRNA levels increased by as much as 4.5-fold after CD40 engagement, other

NF-κB-dependent mechanisms may also contribute to the up-regulation of *pim-1* mRNA by CD40. One such mechanism could be NF-κB-dependent suppression of transcriptional attenuation. Transcriptional attenuation is a situation in which transcription is initiated but the RNA polymerase fails to transcribe to the end of the gene. There is some evidence that transcriptional attenuation limits the production of *pim-1* mRNA in unstimulated cells. In thymocytes, IL-2 has been shown to suppress transcriptional attenuation of the *pim-1* gene and allow read-through transcription (85) such that a full-length transcript is made. Because NF-κB activation has been shown to suppress transcriptional attenuation of the c-*myb* gene in murine erythroleukemia cells (86), it could also be involved in suppressing transcriptional attenuation of the *pim-1* gene. Thus, NF-κB could contribute in multiple ways to the increase in *pim-1* mRNA and Pim-1 protein that is caused by CD40 engagement.

Pim-1 protein levels may also be regulated by posttranscriptional mechanisms. In some cell types, receptor-induced up-regulation of Pim-1 protein levels involves increases in the stability and/or translation rate of the *pim-1* mRNA (46, 87). It is not known whether this also occurs during CD40 signaling in B cells. However, because the anti-CD40-induced increases in *pim-1* mRNA levels are similar in magnitude to the increases in Pim-1 protein levels, posttranscriptional regulatory mechanisms may play only a minor role in the regulation of Pim-1 levels by CD40.

The murine *pim-1* gene gives rise to two isoforms of Pim-1, a 33-kDa protein and a 44-kDa protein. The 44-kDa isoform is an N-terminal extension of the 33-kDa protein that results from translation initiation at an upstream CUG codon (42). Although the two isoforms of murine Pim-1 have comparable kinase activity and can both prevent apoptosis (53, 54), there are some reports that they may function differently. In IL-3-deprived FDCP1 cells, p33 Pim-1 expression prevents apoptosis by cooperating with Bcl-2 while p44 Pim-1 prevents apoptosis in a Bcl-2-independent manner (53). While it is not known whether p44 Pim-1 and p33 Pim-1 have different functions in B cells, we found that the levels of the two isoforms were generally regulated in a similar manner by CD40, BCR, and LPS receptor signaling. Anti-CD40 and LPS treatment up-regulated the expression of both isoforms with similar kinetics while anti-IgM treatment caused an initial transient increase in the expression of both isoforms, followed by the disappearance of both isoforms. Although p44 Pim-1 and p33 Pim-1 were coordinately regulated, the relative abundance of the two isoforms varied somewhat, suggesting that culture conditions or other factors could influence either the use of the two alternate translation initiation sites or the relative stability of the two resulting proteins. Further work is necessary to determine whether changes in the relative abundance of the two Pim-1 isoforms are physiologically significant.

CD40 signaling caused an increase in total Pim-1 kinase activity that was similar in magnitude to the increase in Pim-1 protein level. This suggests that CD40 regulates the level of Pim-1 kinase activity primarily by increasing the level of the Pim-1 protein, as opposed to regulating the specific activity of Pim-1. In either case, our results suggest that CD40 signaling could result in increased phosphorylation of Pim-1 substrates. Several substrates of Pim-1 have been identified, and these include proteins involved in cell cycle progression. Pim-1 has been shown to phosphorylate and activate Cdc25A (88), a phosphatase that promotes cell cycle progression by dephosphorylating negative regulatory sites on cyclin-dependent kinases. Pim-1 also phosphorylates p100, a protein that enhances the activity of c-Myb (89), a transcription factor that promotes the growth and differentiation of hematopoietic cells (90). Other potential substrates of Pim-1 include heterochromatin protein-1γ (91), a protein that may be involved in transcriptional silencing, and PAP-1 (92), a protein of unknown function that binds to Pim-1.

In addition to CD40, we showed that the BCR and the LPS receptor complex also regulate Pim-1 levels in B cells. Moreover, we found that signals emanating from the BCR, CD40, and the LPS receptor complex are integrated at the level of Pim-1 in WEHI-231 cells. Interestingly, how the signals are integrated changes in a temporal manner. At early times (e.g., 2 h), BCR signals amplify the increase in Pim-1 levels that are caused by either anti-CD40 Abs or LPS (see Figs. 6*B* and 7, *C* and *D*). This could reflect the ability of the BCR to activate NF-κB (93). Although BCR signaling increases Pim-1 levels at early times, prolonged BCR signaling (>4 h) causes a sharp decline in Pim-1 levels and also opposes the sustained increases in Pim-1 protein levels that are caused by anti-CD40 Abs or LPS. Thus, the BCR must turn on inhibitory processes that suppress further increases in Pim-1 levels and perhaps also lead to the degradation of existing Pim-1 proteins. These inhibitory processes could include the induction of transcriptional repressors, the induction of proteins that decrease the stability and/or translation of *pim-1* mRNA, or the induction of proteins that decrease the half-life of the Pim-1 protein.

Because Pim-1 can prevent apoptosis and promote cell survival in myeloid cells, we hypothesized that it might also be involved in death vs survival decisions in B cells. Therefore, we analyzed Pim-1 expression in the WEHI-231 cell line in which BCR signaling causes growth arrest and apoptosis that can be prevented by CD40 engagement or LPS treatment. In this system we found that BCR signaling regulated Pim-1 levels in a complex manner in which there was an initial increase in Pim-1 levels followed by a drastic decline. The regulation of a prosurvival factor in this manner is consistent with a model in which BCR signaling initiates an abortive activation program that is replaced by a program of anergy or apoptosis unless the B cell receives costimulatory signals. Consistent with this idea, anti-CD40 Abs and LPS, both of which protect WEHI-231 cells from anti-IgM-induced growth arrest, up-regulated the expression of Pim-1 and opposed the anti-IgM-induced decline in Pim-1 levels. Thus, the up-regulation and/or maintenance of Pim-1 levels correlated with the survival and proliferation of WEHI-231 cells. Further work will test whether Pim-1 expression is either necessary or sufficient to promote the survival of WEHI-231 cells.

Pim-1 could promote B cell survival by acting in concert with c-Myc and c-Myb, two other prosurvival factors that are induced by both CD40L and LPS. Sonenshein and colleagues (94) have shown that c-Myc is a key determinant of life vs death in WEHI-231 cells. Lowering c-Myc levels in various ways results in cell death, whereas maintaining c-Myc levels by expressing an endogenous c-*myc* gene prevents anti-IgM-induced death (95). Interestingly, c-Myc is regulated in an identical fashion to what we have shown for Pim-1. In response to anti-IgM, there is a transient increase in c-Myc levels which is followed by the nearly complete disappearance of c-Myc (95), similar to what we observed for Pim-1. Moreover, CD40L induces the expression of c-Myc and prevents the anti-IgM-induced decline in c-Myc levels (34), as it does for Pim-1. This coordinate regulation of c-Myc and Pim-1 may allow these two protooncogene products to work together to promote cell survival and proliferation, similar to the way that they cooperate to cause pre-B cell lymphomas (58). Although the mechanism underlying this cooperation between c-Myc and Pim-1 is not clear, Cdc25A, a phosphatase that promotes cell cycle progression, is a likely point of convergence. c-Myc is a transcription factor that up-regulates the expression of Cdc25A (96), while Pim-1 phosphorylates Cdc25A and increases its enzyme activity (88). In terms of Cdc25A, Pim-1 can be viewed as a factor that amplifies the

actions of c-Myc. Thus, the role of Pim-1 in CD40- and LPS-induced protection of WEHI-231 cells from anti-IgM-induced growth arrest may be to reduce the amount of c-Myc needed to allow the cells to survive. Conversely, the anti-IgM-induced decrease in Pim-1 levels would make the cells more sensitive to the concomitant decrease in c-Myc levels and thereby promote growth arrest and apoptosis.

In addition to amplifying c-Myc-dependent survival signals, Pim-1 may also amplify survival signals generated by c-Myb, a transcription factor whose expression is induced by CD40 signaling in B cells (97). Pim-1 increases the ability of c-Myb to act as a transcription factor by phosphorylating the p100 coactivator protein which regulates the activity of c-Myb (89). Although few c-Myb-regulated genes have been identified, the ability of v-Myb to transform hematopoietic cells (90) indicates that c-Myb likely regulates the expression of genes involved in survival and proliferation. c-Myb can also cooperate with c-Myc to induce tumors (98). Thus, Pim-1, c-Myb, and c-Myc may constitute a CD40L- and LPS-regulated signaling module that promotes B cell survival and proliferation.

In summary, we have shown that in B cells Pim-1 is regulated by CD40, the BCR, and the LPS receptor complex. Moreover, in WEHI-231 cells, signals emanating from these receptors are integrated such that the resulting Pim-1 protein levels correlate with the survival and proliferation of these cells. Because Pim-1 promotes cell survival and proliferation in other hematopoietic cell types, Pim-1 levels could be a key determinant of B cell survival and proliferation.

Acknowledgments

We thank Linda Matsuuchi for critical reading of the manuscript and for providing many helpful suggestions. We also thank Rebecca Newbury for technical assistance.

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