The inhibitory Fc receptors function to regulate the antigen-driven activation and expansion of lymphocytes. In B cells, the FcγRIIB1 is a potent inhibitor of B cell antigen receptor (BCR) signaling when coligated to the BCR by engagement of antigen-containing immune complexes. Inhibition is mediated by the recruitment of the inositol phosphatase, SHIP, to the FcγRIIB1 phosphorylated tyrosine-based inhibitory motif (ITIM). Here we show that BCR-independent aggregation of the FcγRIIB1 transduces an ITIM- and SHIP-independent proapoptotic signal that is dependent on members of the c-Abl tyrosine kinase family. These results define a novel Abl family kinase-dependent FcγRIIB1 signaling pathway that functions independently of the BCR in controlling antigen-driven B cell responses.

B cell antibody responses are initiated by the binding of multivalent antigens to the B cell antigen receptor (BCR) (1, 2). Engagement of the BCR triggers signal cascades that lead to the proliferation of the B cells and their differentiation into both long and short lived antibody-secreting plasma cells and memory B cells. The antigen-driven expansion and differentiation of B cells is a carefully balanced process that ensures adequate levels of circulating protective antibody and memory B cells and simultaneously avoids excessive deleterious production of antibody such as occurs in autoimmune diseases. The low affinity inhibitory receptor for IgG, FcγRIIB1, has emerged as a key regulator of B cell responses (3). Although B cells express members of the recently identified extended family of FcR homologs these proteins do not appear to bind Fcs and thus do not function as FcRs (4). During an immune response, once antibody levels reach a critical point resulting in the formation of antigen- and antibody-containing immune complexes (ICs), the simultaneous binding of ICs to the BCR and FcγRIIB1 inhibits BCR signaling. Coligation of the FcγRIIB1 and the BCR by ICs leads to the phosphorylation of the FcγRIIB1 by the Src family kinase Lyn on the tyrosines in the immunoreceptor tyrosine-based inhibitory motif (ITIM), resulting in the recruitment of the inositol phosphatase, SHIP, which inhibits the BCR trigger Ca\(^{2+}\) mobilization and B cell proliferation (3). The inhibition of Ca\(^{2+}\) mobilization and proliferation appears to proceed through two separable pathways, one requiring the phosphatase activity of SHIP to hydrolyze PI3K-generated phosphatidylinositol trisphosphate preventing Btk and phospholipase Ca\(^{2+}\) activation (5) and a second requiring SHIP to recruit the Ras GAP-binding protein p62 dok that functions to inhibit extracellular signal-regulated kinase activation (6). The physiological significance of the role of the FcγRIIB1 in regulating BCR signaling is demonstrated by the phenotype of FcγRIIB1-deficient mice that show susceptibility to induced autoimmune diseases and to spontaneous autoimmune disease in certain strains (7).

In addition to its inhibitory activity when coligated to the BCR, the FcγRIIB1, when aggregated to itself, propagates a signal that leads to apoptosis (8) by an ITIM-independent mechanism that is partially blocked by the coligation to the BCR and the recruitment of SHIP (8). Thus, the FcγRIIB1 apparently has the potential to function independently of both ITIM-containing receptors and its own ITIM. The ability of the FcγRIIB1 to induce apoptosis has the potential to control B cell responses at any point in the antigen-driven proliferation and differentiation pathways of B cells when the FcγRIIB1 engages ICs independently of the BCR. For example, the ability of the FcγRIIB1 to induce B cell apoptosis has been proposed to play a role in the maintenance of self-tolerance by the elimination of B cells that lose antigen specificity during the process of somatic hypermutation during germinal center expansion (8). Indeed, FcγRIIB1-deficient mice on certain genetic backgrounds develop autoantibodies and ultimately die of autoimmune glomerulonephritis (7), suggesting that in the absence of the FcγRIIB1 these mice failed to maintain self-tolerance successfully. Alternatively, the engagement of FcγRIIB1 expressed on antibody-secreting, long lived plasma cells that no longer express BCR could serve to eliminate these cells once ICs have reached deleterious levels (9). In either case, BCR-independent ligation of the FcγRIIB1 would play a key role in eliminating potentially harmful antibody responses.

Here we show that BCR-independent aggregation of the FcγRIIB1 initiates a novel c-Abl family kinase-dependent, SHIP- and ITIM-independent signaling pathway that results in cell cycle arrest and apoptosis. Although the transforming capacity of v-Abl in pre-B cells and of BCR-Abl in hematopoietic cells, including the majority of B-acute lymphoid leukemias, is well appreciated (10, 11), a role for the Abl family kinases in normal B cell signaling has been less clearly delineated. The findings presented here describing a role for Abl family kinases in B cell apoptosis may provide further insights into the mechanisms that control both B cell activation and transformation.

**EXPERIMENTAL PROCEDURES**

**Reagents and Cell Lines**—SHIP (M-14 and P1C1) and polyclonal antibody specific for c-Abl (K-12) were purchased from Santa Cruz Biotech-
The Role of c-Abl in FcγRIIB1 Signaling

ology (Santa Cruz, CA). FITC-labeled F(ab\textsuperscript{'})\textsubscript{2} goat antibodies specific for mouse or rabbit IgG, FITC-labeled donkey and goat antibodies specific for mouse IgG, 2.4G2, and biotin-2.4G2 mAbs and mAbs specific for c-Abl (8E9) and cytochrome c were purchased from BD Pharmingen. The phosphotyrosine-specific mAb, 4G10, was purchased from Upstate (Charlottesville, VA). Affinity-purified rabbit antibodies specific for phospho-c-Abl (Tyr\textsuperscript{245}) were obtained from Cell Signaling (Beverly, MA). Immunocomplexes of peroxidase and rabbit antibodies specific for peroxidase (PAP) were purchased from Sigma. Alternatively, ICs were formed by mixing equal amounts of goat IgG and rabbit antibodies specific for the Fc portion of goat IgG (Jackson Immunoresearch Laboratory, West Grove, PA) for 30 min at 4 °C. PP2, piceatan, respectively, ICs were formed by mixing equal amounts of goat IgG and rabbit antibodies specific for peroxidase (PAP) were purchased from Sigma. Alternatively, ICs were formed by mixing equal amounts of goat IgG and rabbit antibodies specific for peroxidase (PAP) were purchased from Sigma. Alternatively, ICs were formed by mixing equal amounts of goat IgG and rabbit antibodies specific for peroxidase (PAP) were purchased from Sigma. Alternatively, ICs were formed by mixing equal amounts of goat IgG and rabbit antibodies specific for peroxidase (PAP) were purchased from Sigma. Alternatively, ICs were formed by mixing equal amounts of goat IgG and rabbit antibodies specific for peroxidase (PAP) were purchased from Sigma. Alternatively, ICs were formed by mixing equal amounts of goat IgG and rabbit antibodies specific for peroxidase (PAP) were purchased from Sigma. Alternatively, ICs were formed by mixing equal amounts of goat IgG and rabbit antibodies specific for peroxidase (PAP) were purchased from Sigma. Alternatively, ICs were formed by mixing equal amounts of goat IgG and rabbit antibodies specific for peroxidase (PAP) were purchased from Sigma. Alternatively, ICs were formed by mixing equal amounts of goat IgG and rabbit antibodies specific for peroxidase (PAP) were purchased from Sigma. Alternatively, ICs were formed by mixing equal amounts of goat IgG and rabbit antibodies specific for peroxidase (PAP) were purchased from Sigma. Alternatively, ICs were formed by mixing equal amounts of goat IgG and rabbit antibodies specific for peroxidase (PAP) were purchased from Sigma. Alternatively, ICs were formed by mixing equal amounts of goat IgG and rabbit antibodies specific for peroxidase (PAP) were purchased from Sigma. Alternatively, ICs were formed by mixing equal amounts of goat IgG and rabbit antibodies specific for peroxidase (PAP) were purchased from Sigma. Alternatively, ICs were formed by mixing equal amounts of goat IgG and rabbit antibodies specific for peroxidase (PAP) were purchased from Sigma. Alternatively, ICs were formed by mixing equal amounts of goat IgG and rabbit antibodies specific for peroxidase (PAP) were purchased from Sigma. Alternatively, ICs were formed by mixing equal amounts of goat IgG and rabbit antibodies specific for peroxidase (PAP) were purchased from Sigma. Alternatively, ICs were formed by mixing equal amounts of goat IgG and rabbit antibodies specific for peroxidase (PAP) were purchased from Sigma. Alternatively, ICs were formed by mixing equal amounts of goat IgG and rabbit antibodies specific for peroxidase (PAP) were purchased from Sigma. Alternatively, ICs were formed by mixing equal amounts of goat IgG and rabbit antibodies specific for peroxidase (PAP) were purchased from Sigma. Alternatively, ICs were formed by mixing equal amounts of goat IgG and rabbit antibodies specific for peroxidase (PAP) were purchased from Sigma. Alternatively, ICs were formed by mixing equal amounts of goat IgG and rabbit antibodies specific for peroxidase (PAP) were purchased from Sigma. Alternatively, ICs were formed by mixing equal amounts of goat IgG and rabbit antibodies specific for peroxidase (PAP) were purchased from Sigma. Alternatively, ICs were formed by mixing equal amounts of goat IgG and rabbit antibodies specific for peroxidase (PAP) were purchased from Sigma. Alternatively, ICs were formed by mixing equal amounts of goat IgG and rabbit antibodies specific for peroxidase (PAP) were purchased from Sigma. Alternatively, ICs were formed by mixing equal amounts of goat IgG and rabbit antibodies specific for peroxidase (PAP) were purchased from Sigma. Alternatively, ICs were formed by mixing equal amounts of goat I

**Intracellular Staining**—For cytochrome c staining, cells (5 × 10\textsuperscript{5}/ml) were fixed by treatment with 2% paraformaldehyde for 15 min at room temperature. The cells were washed and permeabilized by treatment with ice-cold 90% methanol for 30 min on ice. Permeabilized cells were blocked using 50 µg/ml goat IgG in PBS containing 5% fetal bovine serum for 60 min at room temperature. Cells were washed in staining solution (1 × PBS, 5% fetal bovine serum, 0.09% NaN\textsubscript{3}, and 1 µg of cytochrome c-specific mAb or isotype control antibody was added for 1 h at room temperature. After washing, cells were incubated with FITC-conjugated F(ab\textsuperscript{'})\textsubscript{2} goat anti-mouse Ig for an additional 1 h before flow cytometric analysis. For the detection of phospho-c-Abl, intracellular staining was performed according to the directions provided by the manufacturer (Cell Signaling). Cy2- or FITC-conjugated F(ab\textsuperscript{'})\textsubscript{2} goat anti-rabbit Ig was added before flow cytometric analysis.

**Caspase Assays**—Cells (1–2 × 10\textsuperscript{5}/ml) were treated with 1 µl of caspase substrates from CaspGLOW fluorogenic active caspase staining kits (caspases 3, 8, and 9) (BioVision, Mountain View, CA) and incubated for 45–60 min at 37 °C. Cells were washed and analyzed by flow cytometry using the FL-1 channel.

**TUNEL Assay**—A DeadEnd fluorometric TUNEL system (Promega, San Luis Obispo, CA) was used according to the manufacturer’s protocol. Briefly, cells (5 × 10\textsuperscript{5}) were fixed by incubation in 5 ml of 1% ice-cold paraformaldehyde for 20 min. Fixed cells were washed with PBS and resuspended in 5 ml of 70% ice-cold ethanol and kept at −20 °C overnight. Cells were washed and transferred to 1.5-ml microcentrifuge tubes. The pellet was resuspended in 80 µl of equilibration buffer for 5 min at room temperature before centrifugation. The nuclei were then incubated for 1 h at 37 °C in 50 µl of equilibration buffer containing fluoroscein-12-DUTP in the presence of a nucleotides and terminal deoxynucleotidyl transferase to label 3’-OH termini of the strand breaks of fragmented DNA. The reaction was stopped by adding 1 ml of 20 mM EDTA. After washes and centrifugation, the pellet was resuspended in 0.5 ml of propidium iodide solution (5 µg/ml in PBS, 250 µg of RNase A) for 30 min at room temperature before analysis by FACS for DNA breaks (TUNEL) using FL-1 and DNA content (propidium iodide) using FL-3. Data were analyzed by Flowjo, and contour plots (contour levels: 10% probability) were displayed.

**In Vitro Kinase Assays**—Cells (10\textsuperscript{5}) were lysed in cold buffer (0.5% Nonidet P-40, 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 2 mM MgCl\textsubscript{2}, 1 mM Na\textsubscript{2}VO\textsubscript{4}, and CLAP (2.5 mg/ml each chymostatin, leupeptin, antipain, and pepstatin A in dimethyl sulfoxide) for 45 min to 1 h at 37 °C. Lysates were precleared with protein A/G-Sepharose beads at 4 °C for 1 h, and the cleared lysates were incubated with the specific antibody (2 µg) and protein A or G beads (40 µl 50% slurry) at 4 °C overnight. The beads were washed three times with lysis buffer or PBS, and the bound proteins were eluted by boiling for 5 min in SDS sample buffer. The immunoprecipitates were analyzed by 7.5 or 10% SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA), and membranes were incubated with blocking solution (5% nonfat milk or 5% bovine serum albumin in TBS-T wash buffer) containing the specific antibodies at 4 °C overnight. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. The blots were visualized with enhanced chemiluminescence (ECL; Amersham Biosciences) on x-ray films (Kodak).

**Cell Cycle Analysis**—For cell cycle analyses cells (5 × 10\textsuperscript{5} to 10\textsuperscript{6}/ml) were pelleted and resuspended in hypotonic DNA staining solution (30 µg/ml propidium iodide, 0.1% sodium citrate, 0.1% Triton X-100, and 20 µg/ml RNase A) for 4–6 h at 4 °C or for 1 h at room temperature and then analyzed with a FACScan (BD Biosciences) to determine the DNA content. The fraction of hypodiploid (apoptotic) cells was determined by gating on the cells below the G\textsubscript{1}/G\textsubscript{0} peak of DNA content. G\textsubscript{1}/G\textsubscript{0} and G\textsubscript{2}/M populations were determined and quantified using the cell cycle analysis tool of Flowjo software (Tree Star, Inc., Ashland, OR).

**Measurement of Mitochondrial Membrane Potential (MMP) **—Cells (1–2 × 10\textsuperscript{5}/ml) were incubated in the absence or presence of 50–100 µM STI571 and/or 10 µg/ml IC in medium for 4–6 h. Cells were washed with PBS and incubated with 0.5 µg/ml JC-1 (Molecular Probes, Eugene, OR) for 30 min at 37 °C, washed, and analyzed by FACS using channel 1 (JC-1/red) and channel 2 (JC-1/green). Dead cells were gated out from the analysis. In dot blots, the left upper quadrant with high red and low or no green fluorescence represents cells with intact MMP, and the remaining three quadrants represent cells with depolarized MMP, which are manifested by reduced red fluorescence and/or increased green fluorescence.

**RESULTS**

**BCR-independent FcγRIIB1 Aggregation Results in G\textsubscript{1} Cell Cycle Arrest and Apoptosis through a Caspase 3- and 9- and Mitochondria-
dependent Pathway—To determine the effect of FcγRIIB1 aggregation on cell cycle progression in B cells, DT40 chicken B cells that express the mouse FcγRIIB1, DT(FcγR⁺), were treated with the biotinylated FcγRIIB1-specific mAb, 2.4G2 (b-2.4G2), and avidin to cross-link the FcγRIIB1, incubated with propidium iodide to label DNA, and the DNA content was determined by flow cytometry. Untreated cells were unsynchronized and distributed in the G₁/G₀ and G₂/M phases of the cell cycle (Fig. 1A). Cross-linking the FcγRIIB1 resulted in growth arrest and accumulation of cells in the G₁/G₀ phase. Growth arrest in G₁/G₀ was dependent on the dose of the b-2.4G2 mAb but was not dependent on a functional ITIM or on the inositol phosphatase, SHIP, because aggregation of the FcγRIIB1 resulted in cell cycle arrest in DT(FcγR⁺) cells that were deficient in SHIP, DT(FcγR⁺SHIP⁻), or expressed a mutant version of the FcγRIIB1 in which the ITIM tyrosine (residue 309) was mutated to a phenylalanine, DT(FcγR Y309F) (Fig. 1B).

Aggregation of the FcγRIIB1 was shown earlier to induce apoptosis in DT(FcγR⁺) cells as measured by DNA fragmentation (8). As indicated either by an increase in hypodiploid cells measured by propidium iodide staining (data not shown) or TUNEL-positive cells, aggregation of the FcγRIIB1 led to apoptosis (Fig. 1C). Apoptosis was most efficiently induced by incubating the DT(FcγR⁺) cells with ICs (Fig. 1C) rather than the b-2.4G2 mAb and avidin (data not shown) in that within 16–20 h of treatment with ICs apoptosis was evident, and in contrast, apoptosis induced with b-2.4G2 and avidin was only measurable at 36 h post-treatment. Induction of apoptosis, like induction of G₁/G₀ growth arrest, was not dependent on either a functional ITIM or on SHIP because aggregation of the FcγRIIB1 on DT(FcγR Y309F) and DT(FcγR⁺SHIP⁻) cells resulted in apoptosis (Fig. 1C). Cells appeared to enter apoptosis from the G₁/G₀ phase of the cell cycle (Fig. 1D), and this pattern was not dependent on a functional ITIM or on SHIP (data not shown).

Concerning the mechanism by which apoptosis is induced, treatment of DT(FcγR⁺) cells with IC to cross-link the FcγRIIB1 resulted in the induction of the activity of the apoptosis-associated caspases 9 and 3 in ~25% of the cells (Fig. 2A). The number of caspase-positive cells correlated with the number of cells induced to undergo apoptosis indicated by either the number of hypodiploid cells (data not shown) or TUNEL-positive cells (Fig. 1C). Cross-linking the FcγRIIB1 also induced the release of cytochrome c from the mitochondria in DT(FcγR⁺) cells (Fig. 2B). Lastly, FcγRIIB1 aggregation resulted in a Ca²⁺-dependent depolarization of the mitochondria membrane measured using the membrane-permeable lipophilic cationic fluorochrome, JC-1 (13). JC-1 is taken up into mitochondria by the membrane potential where JC-1 associates with the number of cells induced to undergo apoptosis indicated with c-Abl in an ITIM, SHIP, and Src Family Kinase-independent Mechanism—To delineate the signaling cascade triggered by FcγRIIB1 aggregation which leads to apoptosis we first investigated the phosphorylated state of the FcγRIIB1 following cross-linking. DT(FcγR⁺) and DT(FcγR⁺) cells were treated at 4 °C briefly to cross-link the FcγRIIB1 using b-2.4G2 mAb and avidin, warmed to 37 °C, and incubated for 5 min, lysed, and phosphotyrosine-containing proteins were immunoprecipitated from the lysates using the phosphotyrosine-specific mAb, 4G10. The immunoprecipitates were analyzed by SDS-PAGE and immunoblotting probing for the FcγRIIB1 using rabbit antibodies specific for the cytoplasmic domain of FcγRIIB1. The FcγRIIB1 was tyrosine phosphorylated following aggregation of the FcγRIIB1 by b-2.4G2 mAb and avidin (Fig. 3A), and phosphorylation was dependent on the...
concentration of the cross-linking mAb, 2.4G2 (data not shown). Similar results were obtained when cells were treated with ICs; however, receptor phosphorylation was not observed when cells were treated with isotype-matched nonspecific mAbs or with monovalent Fab 2.4G2 (data not shown). Moreover, the Fc/H9253RIIB1 was not present in immunoprecipitates using isotype control antibodies (data not shown).

After coligation of the Fc/H9253RIIB1 to the BCR, the Fc/H9253RIIB1 has been shown to be tyrosine phosphorylated by the Src family kinase, Lyn, on ITIM residues (14). To determine whether phosphorylation of the Fc/H9253RIIB1 after BCR-independent aggregation required the ITIM tyrosine residue, DT(Fc/H9253R Y309F) cells were treated to cross-link the Fc/H9253RIIB1 and tyrosine-phosphorylated Fc/H9253RIIB1 was detected as described above. The FcR Y309F receptor was phosphorylated after Fc/H9253RIIB1 cross-linking (Fig. 3A), and the degree of phosphorylation of the wild type and FcR Y309F appeared similar, suggesting that ITIM tyrosines were not phosphorylated in either the wild type or mutant Fc/H9253RIIB1 after aggregation. In addition to the ITIM tyrosine at position 309 the Fc/H9253RIIB1 contains three tyrosine residues within the cytoplasmic domain at positions 264, 290, and 326 (12). As will be shown below, in mouse A20 cells expressing a truncated form of the Fc/H9253RIIB1 which ends at position 289 (FcYΔ289) the receptor is phosphorylated after BCR-independent aggregation (see Fig. 7), suggesting that tyrosine 264 may be the target of phosphorylation.

The Fc/H9253RIIB1 was also phosphorylated in DT(FcR+SHIP−), and the level of phosphorylation of the Fc/H9253RIIB1 appeared similar to that in DT40(FcR+SHIP−) cells (Fig. 3A), suggesting that SHIP is not required for Fc/H9253RIIB1 phosphorylation and, moreover, may not regulate the signaling cascade triggered by aggregation of the Fc/H9253RIIB1 at the level of receptor phosphorylation.

In an attempt to identify the kinase activity responsible for the phosphorylation of the Fc/H9253RIIB1, cells were treated with several inhibitors that blocked the activities of known components of B cell signaling pathways including Lyn, Syk, Btk, p38, c-Jun N-terminal kinase, and PI3K. However, none of the inhibitors was effective in blocking Fc/H9253RIIB1 phosphorylation (data not shown), suggesting that the Fc/H9253RIIB1 triggered a novel BCR-independent signaling pathway. Because the Abl family kinases have been implicated in proapoptotic signaling (15) they were potential candidates to play a role in Fc/H9253RIIB1 phosphorylation. To determine whether c-Abl associated with the Fc/H9253RIIB1 following BCR-independent aggregation immunoprecipitates of c-Abl from

**FIGURE 2.** FcγRIIB1 aggregation induces caspase activation, cytochrome c release, and mitochondrial membrane depolarization. A, 10⁶/ml DT(FcR−) and DT(FcγR+) cells were incubated for 20 h in medium alone or in medium containing 5 μg/ml ICs. Caspase substrates were added to the medium 30 min before harvesting the cells, and the caspase activities were determined by flow cytometry. B, DT(FcγR−) and DT(FcγR+) cells were incubated in medium alone or in medium containing ICs for 7–8 h. Cytoplasmic cytochrome c was measured by intracellular staining using cytochrome c-specific antibodies or isotype-matched antibodies. C, DT(FcγR−) and DT(FcγR+) cells were incubated in medium alone or in medium containing ICs for 4–5 h. Cells were washed and incubated with JC-1 for 30 min at 37°C. The percentage of DT(FcγR−) and DT(FcγR+) cells showing depolarized MMP in four independent experiments is given.
The Role of c-Abl in FcγRIIB1 Signaling

DT(FcγR⁺), cells treated to cross-link the FcγRIIB1 were analyzed by SDS-PAGE and immunoblotting probing for FcγRIIB1. FcγRIIB1 was present in c-Abl immunoprecipitates from cells in which the FcγRIIB1 was cross-linked and phosphorylated but not from untreated cells (Fig. 3A). The FcγRIIB1 appears as a band in the immunoblots immediately above the band present in all lanes representing the rabbit antibodies used to immunoprecipitate c-Abl. The association of c-Abl with the FcγRIIB1 was dependent on the dose of the b-2.4G2 mAb and was also induced using ICs to aggregate the FcγRIIB1 (data not shown). The association of c-Abl with the phosphorylated FcγRIIB1 was not dependent on the ITIM tyrosine and was not significantly regulated by SHIP and as indicated by the association of c-Abl with the FcyRIIB1 in DT(FcγY Y309F) and DT(FcγY⁺ SHIP⁻) cells (Fig. 3A).

Using an antibody specific for phosphorylated c-Abl it was possible to show by flow cytometry that aggregation of the FcγRIIB1 resulted in the phosphorylation of c-Abl (Fig. 3B). c-Abl phosphorylation was not dependent on ITIM tyrosine or SHIP because c-Abl was phosphorylated in DT(FcγY Y309F) cells and in DT(FcγY⁺ SHIP⁻) cells in which the FcγRIIB1 was cross-linked (Fig. 3B).

The Phosphorylation of FcγRIIB1, Its Association with c-Abl, and Induction of Apoptosis Are Dependent on Both c-Abl and Arg Kinase Activities—To determine whether phosphorylation of the FcγRIIB1, its association with c-Abl, and induction of apoptosis were dependent on the activity of the c-Abl, DT(FcγR⁺) cells were treated with the Abl inhibitor, STI571 (15) prior to aggregation of the FcγRIIB1. STI571 blocked both the phosphorylation of the FcγRIIB1 and the association of c-Abl with the FcγRIIB1 in a STI571 dose- and time-dependent fashion (Fig. 4A). The concentration of STI571 required to block FcγRII phosphorylation is similar to that reported by others to block c-Abl-induced apoptosis of B cells as measured by depolarization of the mitochondrial membrane (Fig. 4B). Cross-linking the FcγRIIB1 induced ~35% of cells to undergo apoptosis, and this number was reduced significantly in a STI571 dose-dependent fashion.

To evaluate directly the requirement for c-Abl in FcγRIIB1 signaling for apoptosis, the ability of FcγRIIB1 cross-linking to induce FcγRIIB1 phosphorylation in DT40 cells expressing the FcγRIIB1 in which the c-Abl gene was deleted, DT(FcγY⁺ Abl⁻), was tested. FcγRIIB1 clustering-induced phosphorylation was only partially blocked in DT(FcγY⁺ Abl⁻) cells (data not shown). Because ST1571 blocked receptor phosphorylation completely in wild type cells this observation suggested that the activity of c-Abl may be partially redundant with that of another Abl family kinase. DT40 cells that expressed the FcγRIIB1 and were deficient in both c-Abl and Arg, DT(FcγY⁺ Abl⁻ Arg⁻), did not phosphorylate the FcγRIIB1 or undergo apoptosis in response to FcγRIIB1 cross-linking (Fig. 4, C and D). Arg was also detected associated with the FcγRIIB1 after FcγRIIB1 cross-linking (Fig. 4C). Taken together these results indicate a direct role for the Abl family kinases, c-Abl and Arg, in FcγRIIB1-induced apoptosis.

The effect of FcγRIIB1 cross-linking on Abl kinase activity was determined. DT(FcγY⁺) cells were treated to aggregate the FcγRIIB1, the cells were lysed, Ab kinases were immunoprecipitated, and the immunoprecipitates were assayed for Abl kinase activity using the Abl substrate Crk. Compared with untreated cells, the immunoprecipitated Abl kinase activity was increased in cells in which the FcγRIIB1 was cross-linked (Fig. 5A). The increase in Abl kinase activity was not observed in cells treated to cross-link the BCR alone using F(ab')₂, antibodies specific for Ig or treated with intact, Fc-containing, antibodies specific for Ig that function to coligate the BCR and the FcγRIIB1 (Fig. 5A), suggesting that BCR-dependent signaling pathways do not involve Abl.

To determine whether the cytoplasmic domain of the FcγRIIB1 served as substrate for Abl kinases, the cytoplasmic domain of the FcγRIIB1 expressed as a glutathione S-transferase fusion protein was incubated with purified c-Abl or Arg. Both c-Abl and Arg phosphorylated the FcγRIIB1 cytoplasmic domain in vitro, and the phosphorylation was blocked by STI571. Thus, the cytoplasmic domain of the FcγRIIB1 appears to be a substrate for Abl kinases in vitro, suggesting that Abl kinases may function to phosphorylate the FcγRIIB1 directly in vivo.

BCR-dependent versus -independent Aggregation of the FcγRIIB1 Leads to Distinct Signaling Pathways—Taken together the results presented thus far indicate that BCR-independent aggregation of the FcγRIIB1 induces a signaling pathway that is distinct from the BCR-dependent FcγRIIB1 signaling pathway in that it involves Abl kinases and is both ITIM- and SHIP-independent. The BCR-dependent and -independent signaling pathways were compared directly in DT(FcγY⁺)}.
The Role of c-Abl in FcγRIIB1 Signaling

FIGURE 4. FcγRIIB1-induced receptor tyrosine phosphorylation and apoptosis is dependent on c-Abl and Arg. A, DT(FcγR1) cells were untreated or pretreated with graded doses of STI571 (10, 25, 50, and 100 µM) for 1 or 3 h before treatment with 5 µg/ml 2-B-4G2 plus 3 µg/ml avdin for 5 min at 37 °C. The cells were lysed and lysates subjected to immunoprecipitation (IP) with antibodies specific for phosphoryrosine (4G10), c-Abl (K-12), or FcγR (2.4G2). The immunoprecipitates were analyzed by SDS-PAGE and immunoblotting (WB) probing for FcγR. The arrow indicates the position of the immunoprecipitating antibody (lg). B, cells were incubated in medium alone or in medium containing STI571 for 30 min before incubating with 10 µg/ml ICs for 4–5 h at 37 °C. Cells were washed and loaded with the membrane-permeable lipophilic cationic fluorochrome, JC-1 (0.5 µg/ml) and incubated at 37 °C for 30 min and then analyzed by flow cytometry as described under “Experimental Procedures.” The percentage of cells with depolarized MMP was measured, and the average results from four independent experiments are given. C, DT(FcγR1) and DT(FcγR1c- Arg) cells, expressing similar levels of FcγRIIB1 as measured by flow cytometry, were treated as above and subject to immunoprecipitation with antibodies specific for phosphoryrosine (4G10) or Arg and immunoblotting probing for FcγR or Arg. D, DT(FcγR1) and DT(FcγR1c- Abl- Arg) cells were untreated or treated with 5 µg/ml ICs for 20 h. Cells were harvested for propidium iodide staining, and the sub-G1/G0 proportion was measured and shown as percent apoptotic cells.

as has been shown previously, after coligation of the BCR and FcγRIIB1 using intact, Fc-containing Ig-specific antibodies, the FcγRIIB1 becomes phosphorylated as it does after BCR-independent aggregation using FcγR-specific mAb (Fig. 6). However, phosphorylation of the FcγRIIB1 after coligation of the BCR and FcγRIIB1 was sensitive to inhibition by the Src family kinase inhibitor PP2 in contrast to BCR-independent FcγRIIB1 aggregation-induced phosphorylation that showed no sensitivity to PP2. Moreover, the association of c-Abl with the FcγRIIB1 was not sensitive to PP2, and the BCR-dependent aggregation of the FcγRIIB1 did not induce the association of c-Abl with the FcγRIIB1 (Fig. 6).

To further compare signaling after BCR-independent FcγRIIB1 cross-linking versus FcγRIIB1 coligation to the BCR the mouse A20 B cell line, IIA.6, which does not express an endogenous FcγRIIB1 but which has been transfected with either the wild type mouse FcγRIIB1, IIA.1.6 (FcγR1) or a truncated version of the receptor that ends at residue 289, IIA.1.6 (FcγRΔ289), and consequently lacks the ITIM tyrosine at 309 (12), was analyzed. Cross-linking the FcγRIIB1 in IIA.1.6 (FcγR1) cells using biotinylated 2.4G2 mAb plus avidin resulted in a 3.4–4-fold increase in apoptosis in three separate experiments. The increase in apoptosis was reflected in a decrease in the G1 phase of the cell cycle, similar to that observed for DT40 cells (Fig. 1). The results of the analysis showed that FcγRIIB1 triggers either an ITIM- and SHIP-independent signaling cascade that depends on Abl or a Src kinase-, ITIM-, and SHIP-dependent pathway determined by whether it is aggregated independently of the BCR or coligated with the BCR. As shown in Fig. 7A, BCR-independent aggregation of the wild type FcγRIIB1 in the IIA.1.6 (FcγR1) cells using b-2.4G2 antibody and avidin resulted in the phosphorylation of the FcγRIIB1 and its association with c-Abl. The FcγRIIB1 was also present in SHIP immunoprecipitates after BCR-independent FcγRIIB1 aggregation (Fig. 7A), but significantly, the SHIP was not phosphorylated in these cells (Fig. 7B). Coligation of the wild type FcγRIIB1 and the BCR in the IIA.1.6 (FcγR1) cells using anti-Ig resulted in the phosphorylation of the FcγRIIB1 (Fig. 7A). As indicated, the immunoprecipitates from anti-Ig-treated cells contained the rabbit anti-Ig that was detected by the goat antibodies specific for rabbit Ig used to develop the immunoblots. However, the FcγRIIB1 did not associate with c-Abl after coligation (Fig. 7A). The FcγRIIB1 associated with SHIP and the SHIP in anti-Ig-treated cells was phosphorylated (Fig. 7B).

BCR-independent aggregation of the IIA.1.6 (FcγRΔ289) using b-2.4G2 and avidin resulted in its phosphorylation and association with c-Abl (Fig. 7A), indicating that c-Abl bound to the FcγRIIB1 independently of the ITIM and the N-terminal fragment of the cytoplasmic domain. The aggregation of the FcγRΔ289 also resulted in the association of the receptor with SHIP (Fig. 7A), but SHIP was not phosphorylated in these cells (Fig. 7B). Thus, SHIP appears to associate with the phosphorylated FcγRIIB1:Abl complex in an inactive form and independently of the ITIM. Coligation of the FcγRΔ289 with the BCR using anti-Ig antibodies did not result in receptor phosphorylation, nor the association of the receptor with Abl or SHIP (Fig. 7A). Although the FcγRΔ289 does not associate with SHIP in immunoprecipitates, SHIP is phosphorylated after coligation of the FcγRΔ289 and BCR in this cell line (Fig. 7B). Taken together, these findings suggest that the FcγRIIB1 activates either a c-Abl-dependent, ITIM- and SHIP-independent pathway after BCR-independent aggregation or a SHIP- and ITIM-dependent, c-Abl-independent pathway after coligation with the BCR. Even
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DISCUSSION

c-Abl is a clinically important tyrosine kinase the activity of which is dysregulated in a variety of hematopoietic cancers by Philadelphia chromosome translocations (11). Indeed the c-Abl inhibitor STI751 is proving to have significant efficacy in treating chronic myelogenous leukaemia. However, the role of c-Abl in normal lymphoid signaling is not well understood. Recent studies implicate c-Abl in ligand-independent antigen receptor signaling during both B and T lymphocyte development. From studies using the Abl kinase inhibitor STI571, c-Abl has been suggested to play a role in the activation of Ig light chain genes in the transition from pre-B to immature B cell stage through its inhibition of the genes encoding the transcription factors Spi-B and IRF-4 (16). In Jurkat T cell line treatment with STI517 increased RAG-1 expression, suggesting a role for Abl kinases in the tonic signaling pathways in T cells that suppress RAG gene expression (17). However, a role for Abl kinases in mature B lymphocyte signaling has not been established.

though SHIP does not appear to participate in the BCR-independent aggregation-induced signaling it remains associated with the aggregated FcγRIIIB1 complex in an inactive state.

The FcγRIIIB1 is a well characterized inhibitory receptor that functions in B cells to regulate the antigen-driven activation and expansion of cells. When coligated to the BCR the FcγRIIIB1 blocks BCR signaling (3) and when aggregated independently of the BCR, the FcγRIIIB1 has been shown to induce apoptosis by an ITIM-independent mechanism (8). Here we provide evidence that FcγRIIIB1 aggregation triggers a signaling cascade in which c-Abl family kinases play an essential early role. Significantly, this pathway appears to be distinct from pathways that predominate when the FcγRIIIB1 are coligated to the BCR. BCR-independent FcγRIIIB1 aggregation leads to the phosphorylation of the receptor and the induction of cell cycle arrest and apoptosis in an ITIM- and SHIP-independent fashion. This is in contrast to the ITIM- and SHIP-dependent FcγRIIIB1 signaling induced after coligation with the BCR. Indeed, the Abl kinase inhibitor, STI571, blocks BCR-independent FcγRIIIB1 signaling but has no effect on signaling triggered by coligation of the BCR and FcγRIIIB1.

These results suggest the existence of an early molecular switch that ultimately engages the aggregated FcγRIIIB1 with c-Abl and the FcγRIIIB1 coligated with the BCR. An unanswered question is under what conditions and in which B cell subsets would BCR-independent versus BCR-dependent signal predominate? In the studies described here B cells were exposed to reagents that would either engage the FcγRIIIB1 alone or coligate the FcγRIIIB1 and the BCR. However, under physiological conditions it is reasonable to assume that B cells would encounter both ICs that contain the B cell antigen and those that do not. Thus, both BCR-dependent and -independent FcγRIIIB1 aggregates would initially form, and the signaling outcome would likely depend on the relative concentrations of the specific and nonspecific ICs. If so, this suggests that the c-Abl and SHIP not only

FIGURE 5. c-Abl and Arg phosphorylate the cytoplasmic domain of the FcγRIIIB1 in vitro. A, DT(FcγR-)/ cells were untreated (lane 1) or treated with either b-2.4G2 mAb and avidin to cross-link the FcγRIIIB1 (lane 2), with intact rabbit antibodies specific for mouse Ig (S μg/ml) to coligate the FcγRIIIB1 and the BCR (lane 3), or with f(ab′)2, fragments of Ig-specific antibodies to cross-link the BCR alone (lane 4) for 5 min at 37 °C. Cells were lysed, and lysates were immunoprecipitated (IP) with mAb specific for c-Abl (BE9). The immunoprecipitates were washed and used in vitro kinase reactions with Crk as the substrate. A recombinant c-Abl was used as a positive control (lane 5). The proteins were eluted by SDS sample buffers, fractionated by SDS-PAGE, and immunoblotted (WB) with antibodies specific for phospho-Crk or c-Abl. The substrate input of Crk was visualized by Ponceau S staining. B, purified c-Abl and Arg proteins were incubated with glutathione S-transferase fusion proteins containing the cytoplasmic domain of FcγRIIIB1 in the absence or presence of 10–100 μM STI571 at 37 °C for 1 h. The reactions were terminated by adding SDS sample buffers and boiled for 5 min before SDS-PAGE analysis and immunoblotting with an anti-phosphotyrosine antibody (4G10). The substrate input of the cytoplasmic domain of the FcγRIIIB1 fusion proteins was visualized by Ponceau S staining.

FIGURE 6. The FcγRIIIB1 BCR-dependent and -independent signaling pathways differ in their dependence on Src family kinases and association with c-Abl. DT(FcγR-)/ cells were untreated or pretreated with PP2 before the addition of either b-2.4G2 mAb and avidin to cross-link the FcγRIIIB1 or intact rabbit antibodies specific for mouse Ig (5 μg/ml) to coligate the FcγRIIIB1 and the BCR for 5 min at 37 °C. Cells were lysed and the lysates subjected to immunoprecipitation (IP) using the phosphotyrosine-specific mAb 4G10, c-Abl-specific antibodies, or the FcγR-specific mAb 2.4G2. The immunoprecipitates were analyzed by SDS-PAGE and immunoblotting (WB) probing for FcγRIIIB1 or for c-Abl. The IgH chain of the rabbit antibodies used to coligate the BCR and FcγRIIIB1 (lg) indicated by an arrowhead is detected in the immunoblot by the goat antibodies specific for rabbit Ig used to develop the immunoblot. The positions of the FcγRIIIB1(Fc) are shown by arrows.
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**ACKNOWLEDGMENTS**—We are grateful to Dr. John Cambier (National Jewish Hospital, Denver, CO) for graciously providing the A20 cells lines and the FcγRIIIB1-specific antibodies used for these studies.
The B Cell Inhibitory Fc Receptor Triggers Apoptosis by a Novel c-Abl Family Kinase-dependent Pathway
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J. Biol. Chem. 2005, 280:35247-35254.
doi: 10.1074/jbc.M505308200 originally published online August 22, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M505308200

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