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c-Abl is a nonreceptor tyrosine kinase that we have recently linked to growth factor receptor signaling. The c-Abl kinase is ubiquitously expressed and localizes to the cytoplasm, plasma membrane, cytoskeleton, and nucleus. Thus, c-Abl may regulate signaling processes in multiple subcellular compartments. Targeted deletion or mutation of c-Abl in mice results in a variety of phenotypes, including splenic and thymic atrophy and lymphopenia. Additionally, lymphocytes isolated from specific compartments of c-Abl mutant mice have reduced responses to a variety of stimuli and an increased susceptibility to apoptosis following growth factor deprivation. Despite these observations, little is known regarding the signaling mechanisms responsible for these phenotypes. We report here that splenic B cells from c-Abl-deficient mice are hyporesponsive to the proliferative effects of B cell Ag receptor (BCR) stimulation. The c-Abl kinase activity and protein levels are elevated in the cytosol following activation of the BCR in B cell lines. We show that c-Abl associates with and phosphorylates the BCR coreceptor CD19, and that c-Abl and CD19 colocalize in lipid membrane rafts. These data suggest a role for c-Abl in the regulation of B cell proliferation downstream of the BCR, possibly through interactions with CD19. The Journal of Immunology, 2000, 165: 6872–6879.

The c-Abl protein is a highly regulated nonreceptor tyrosine kinase in vivo (1, 2). Structural alterations of the c-abl gene give rise to oncogenic forms of Abl that have been identified in murine, feline, and human leukemias (2). The c-Abl protein is comprised of multiple domains including Src homology (SH) 3, SH2, and SH1 (catalytic) domains, proline-rich sequences that bind SH3-containing molecules, nuclear localization and export signals, and DNA and actin binding domains (3). The c-Abl kinase is ubiquitously expressed and localizes to the cytosol, membrane, cytoskeleton, and nucleus. Both the localization and kinase activity of c-Abl are regulated within the cell (3). Recently, we uncovered a role for c-Abl downstream of receptor tyrosine kinases (4). We showed that c-Abl is activated by both epidermal growth factor and platelet-derived growth factor in fibroblasts. Activation of c-Abl by growth factors is partially dependent upon the activity of Src family kinases that can directly phosphorylate and activate the c-Abl kinase. Significantly, c-Abl is required for cytoskeletal reorganization in response to platelet-derived growth factor. These studies indicate that c-Abl is an important biological transducer of signals generated by membrane receptors.

Two strains of c-Abl-deficient mice have been generated. The ablnull mice have a targeted deletion of the c-abl gene such that the animal is a complete c-abl null, whereas the ablm1 mice have a targeted carboxyl-terminal truncation that produces a kinase-active protein, lacking binding sites for SH3-containing adaptors and DNA and actin binding domains (5, 6). Interestingly, both lines of mice exhibit a variety of similar phenotypes, including high perinatal lethality, running, abnormal cranial and splenic architecture, and an increased susceptibility to infection (3, 5, 6). Both lines of c-Abl-deficient mice exhibit splenic and thymic atrophy, and are lymphopenic (5, 6). Interestingly, B cells derived from the spleen, but not the bone marrow, of the ablm1 mice exhibit a severe reduction in the ability to form colonies in agar in response to LPS compared with wild-type controls (7). However, this reduction was not observed in liquid culture assays (7). Similarly, the proliferative response to anti-IgM stimulation in liquid culture was reduced in splenocytes from ablm1 mice an average of 50% compared with that from wild-type controls (7). Additionally, progenitor B cell lines derived from the ablm1 mice are more sensitive to apoptosis induced by IL-7 deprivation than lines isolated from wild-type littermates (8). Overall, the phenotype of the c-Abl mutant mice suggests a role for c-Abl in transducing signals in B lymphocytes. However, the role of c-Abl in mediating signaling events in B cells has not been examined.

Various transmembrane molecules regulate B cell activation and signaling. Among these is the B cell Ag receptor (BCR), a multimeric complex composed of the surface Ig and associated CD79α and β subunits (9). Upon clustering of the BCR, Lyn and other Src family kinases are activated to initiate downstream signaling events (9, 10). Numerous signaling events then follow, including amplification of Src family kinase activation, stimulation of the Syk and Btk tyrosine kinases, activation of the Ras/mitogen-activated protein kinase (MAPK) pathway, and increased calcium flux (9–11). These events lead to gene transcription and regulation of B cell proliferation and differentiation.
In addition to the core BCR complex, a number of other cell surface proteins regulate BCR signal transduction. One such protein is CD19, a 95 kDa membrane-spanning glycoprotein expressed only by B cells, from the pre-B cell stage until plasma cell differentiation, and by follicular dendritic cells (12, 13). Aggregation of CD19 alone can lead to signal generation (12, 13). Coaggregation of CD19 with the BCR reduces the threshold for B cell activation by the BCR, indicating that CD19 is a costimulatory molecule in this context (12, 14). Furthermore, CD19 expression is critical for regulating B cell-signaling events. B cells from CD19-deficient mice exhibit reduced tyrosine phosphorylation, phosphoinositide turnover, and proliferation following BCR activation (11, 12, 15, 16). By contrast, overexpression of CD19 in transgenic mice results in hyperresponsiveness to transmembrane signals (12). Thus, CD19 is an important regulator of signaling in B cells.

The CD19 protein contains two C2 Ig-like domains and a ~240 amino acid cytoplasmic tail that is tyrosine phosphorylated at multiple sites in response to BCR activation (12, 13). Phosphorylation of these sites regulates protein interactions that are critical for BCR signaling. However, the protein tyrosine kinases phosphorylating CD19 have not been clearly identified. Recently, Lyn was shown to directly phosphorylate CD19 in vitro and in vivo (11, 17). However, CD19 contains nine distinct potential sites of tyrosine phosphorylation, so it is possible that multiple protein tyrosine kinases phosphorylate CD19. Interestingly, CD19 contains a consensus c-Abl phosphorylation site and two potential binding sites for the c-Abl SH2 domain (18, 19). Herein, we show that CD19 is both a substrate and binding partner for the c-Abl kinase. Furthermore, we demonstrate that the levels of c-Abl tyrosine kinase are elevated in the cytosol following BCR engagement. Significantly, B cells derived from abl−/− knockout mice exhibit an impaired proliferative response following BCR engagement compared with cells derived from wild-type mice. Our results establish that c-Abl is a downstream target of the BCR complex and may modulate BCR signaling events via interactions with CD19.

Materials and Methods

Animals, cell culture, plasmids, and Abs

c-abl (mice (C57Bl6/129SvEv hybrid background) heterozygous for targeted deletion of the c-abl gene were the generous gift of Stephen Goff (Columbia University, New York, NY) (6). Mice were bred and housed in the Duke University vivarium barrier facility. CD-1 outbred mice were obtained from Charles River Breeding Laboratories (Wilmington, MA). Mice were genotyped by PCR.

Ramos and Raji Burkitt lymphoma B cells lines were cultured in RPMI 1640 medium (Life Technologies, Grand Island, NY) containing 10% FBS (Life Technologies). 293T and Bosc23 cells were maintained and transfected as previously described (20, 21). Plasmids encoding the GST, GST-Crk, and GST-Ab SH2 domain proteins have been previously described (4, 22). pSRa-c-Abl and pSRa-c-AblK290R plasmids, encoding wild-type and kinase-inactive c-Abl, respectively, and pMT2CD19 have been previously described (20, 23). GST-CD19 constructs were produced by cloning into the pGex3X vector (Promega, Madison, WI).

Polyclonal antisera 5381 and 5382 against CD19 were generated in New Zealand White rabbits with GST fusions of CD19 amino acids 362–468 (amino acid composition based on human CD19 amino acid sequence reviewed in Ref. 12) as immunogens, respectively. Anti-protein kinase Cβ1 mouse mAb was purchased from Transduction Laboratories (Lexington, KY).

Isolation of primary B lymphocytes and proliferation assays

For preparation of B cells, spleens were removed from wild-type and abl−/− knockout mice (6–12 wk of age) and made into single-cell suspensions. RBC were lysed by washing with 17 mM Tris-HCl, pH 7.4, with 144 mM NH4Cl, and the remaining cells were resuspended in RPMI 1640 medium containing 5% FBS and 2.75 × 10−3 M 2-ME. T cells were depleted using anti-Thy1.2 Ab-coupled magnetic beads (Dynal, Great Neck, NY), and splenocytes were plated in 24-well dishes (1 × 106/well) and incubated with increasing amounts of goat anti-mouse IgM F(ab)2 Ab fragments (Cappel Icn, Costa Mesa, CA) or LPS (Sigma, St. Louis, MO) for 72 h. [3H]Thymidine (1 μCi/ml, 6.7 Ci/mmol; New England Nuclear, Boston, MA) was added to the cultures for the last 17 h. Cells were collected using a PHD cell harvester, and [3H]thymidine incorporation was measured by scintillation counting in a Beckman scintillation counter.

GST fusion proteins and pulldown assays

GST fusion proteins were prepared from bacteria as previously described except that GST-CD19 tail (amino acids 362–540) was prepared in buffers lacking Triton X-100 but containing 0.5% n-dodecyl-β-d-maltoside (Ultrapure Grade; Calbiochem, La Jolla, CA) (21, 22). For GST pulldown assays, Ramos cells were unstimulated or stimulated with goat anti-human IgM F(ab)2 Ab fragments (1:100; BioSource International, Camarillo, CA), lysed, and analyzed for binding to the GST-Ab SH2 domain as previously described (21, 22).

Kinase assays

Ramos or Raji cells (2 × 105 cells/ml) were stimulated with anti-IgM fragments as above or with LPS (100 μg/ml) and immediately washed twice with ice cold hypotonic buffer (Buffer C) (24) containing 1 mM sodium vanadate. Cells were resuspended in three volumes of Buffer C containing a cocktail of protease and phosphatase inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, 1 mM sodium vanadate, 2 mM sodium pyrophosphate, and 25 mM β-glycerophosphate, pH 7.5) and lysed by passage through a 25-gauge needle. Nuclei and insoluble components were removed by centrifugation (24). The remaining soluble, cytoplasmic fraction (50 μg) was diluted into Triton lysis buffer (4) containing protease and phosphatase inhibitors. c-Abl was immunoprecipitated with the monoclonal anti-Ab Ab Ab-3 (Oncogene Research Products, Cambridge, MA). Samples were washed, and c-Abl kinase activity was measured as previously described (4) except that kinase assays were incubated for 20 min at room temperature. For CD19 in vitro phosphorylation assays, c-Abl was immunoprecipitated with Ab-3 or K12 (Santa Cruz Biotechnology, Santa Cruz, CA) from 293T cells transfected with the pSRα-c-Abl or pSRα-c-AblK290R plasmids. c-Abl kinase assays were performed as above.

For mass spectrometric analysis, in vitro phosphorylation of CD19 was performed as above except that cells were lysed in kinase lysis buffer (4) lacking all detergents, but containing 0.5% n-dodecyl-β-D-maltoside, and immunoprecipitates were washed four times with lysis buffer and twice with kinase buffer. Kinase assays were performed as above except the 1 mM cold ATP was used in the absence of [γ-32P]ATP and reactions were at 37°C for 30 min.

Immunoprecipitation and Western blotting

Transfected Bosc23 cells were lysed in a buffer of 50 mM Tris-HCl, pH 8.0; 0.5% Nonidet P-40; 150 mM NaCl; and 2 mM EDTA or RIPA buffer containing the inhibitors described above. Ramos cells were stimulated as above and lysed with a 1% Triton X-100 buffer containing 6 mM HEPES, pH 7.5; 7.5 mM Tris-HCl, pH 7.5; 130 mM NaCl; 15 mM KCl; 3 mM MgCl2; 1 μM CaCl2; 1 mM DTT; and protease and phosphatase inhibitors as above. Lysates were immunoprecipitated with the indicated Abs or controls, and immunocomplexes were washed three to six times with lysis buffer. Western blotting for c-Abl was performed with the anti-Ab Ab SE9 (PharMingen, San Diego, CA). CD19 protein was detected with a mixture of the anti-CD19 antiserum 5381 and 5382. Phosphoryosine was detected using a mixture of the anti-phosphoserine Abs PY99 (Santa Cruz Biotechnology) and 4G10 (Upstate Biotechnology, Lake Placid, NY).

CD19 phosphorylation mapping by mass spectrometry

The phosphorylation of CD19 by c-Abl was determined by analyzing aliquots of the kinase reaction by liquid chromatography/mass spectrometry. The system consisted of an HP1090 liquid chromatograph (Hewlett-Packard, Palo Alto, CA), a 250 μm id × 15 cm Poros R2 (PerSeptive Biosystems, Cambridge, MA) reversed-phase capillary column, and an API-III triple quadrupole mass spectrometer (PE Sciex, Thornhill, Ontario, Canada) equipped with electrospray ionization. For phosphorylation site identification, phosphopeptides were isolated from solution tryptic or Asp-N digests of c-Abl-phosphorylated CD19 using immobilized metal-ion affinity chromatography (25–27). Aliquots of digests were loaded onto a 250 μm id × 2.5 cm C2 or C18 column (Vydac, Hesperia, CA) equilibrated with 50 mM MES buffer, pH 5.5, containing 1 M NaCl. After extensive washing with MES buffer, retained phosphopeptides were eluted and collected with 500 mM ammonium bicarbonate, pH 8.5. Isolated phosphopeptides were desalted.
CD19 IS A SUBSTRATE AND BINDING PARTNER FOR THE C-ABL KINASE

using small reversed phase pipet tip microcolumns and loaded into nanoelectrospray needles (Protana A/S, Odense, Denmark). Positive ion nanoelectrospray mass spectra were acquired using a quadrupole time-of-flight mass spectrometer (Micromass, Manchester, U.K.) fitted with the Z-spray ion source. Enriched phosphopeptides were sequenced by tandem mass spectrometry, allowing specific phosphorylation site identification.

Isolation of lipid membrane rafts

Lipid rafts were isolated as previously described (28). Briefly, Ramos cells were stimulated as above and lysed in 1 ml 0.05% Triton X-100 in TNE (25 mM Tris-HCl, pH 7.6; 150 mM NaCl; 5 mM EDTA; 30 mM sodium pyrophosphate; 10 mM β-glycerophosphate; 1 mM sodium orthovanadate; and protease inhibitors as above). Lysates were diluted with an equal volume of 80% sucrose in TNE and transferred into an ultracentrifuge tube. The lysates were overlaid with 7 ml 30% sucrose in TNE and then with 4 ml 5% sucrose in TNE. The samples were centrifuged 17 h at 200,000 X g at 4°C. The rafts were isolated from the interface between the 5 and 30% sucrose layers and solubilized by the addition of n-octyl β-D-glucopyranoside to 50 mM. The soluble fraction at the bottom of the tube was also collected.

Results

Proliferation downstream of the BCR is reduced in c-Abl-null B cells

To determine whether complete deletion of c-Abl alters BCR-induced cell proliferation we assayed the IgM-induced proliferative response of splenic B cells from wild-type and abl-2 knockout (complete c-Abl-null) mice. Purified splenic B cells were incubated with increasing amounts of anti-IgM F(ab’)2, Ab fragments, and proliferation was determined by measuring [3H]thymidine incorporation (Fig. 1A). The proliferative capacity of c-Abl-null splenic B cells was reduced (43 to 73%) compared with that of cells from wild-type littermates. This difference was observed over an Ab range of 10–160 μg/ml. The reduced proliferative capacity of the c-Abl-null cells was specific for BCR activation, as LPS-induced thymidine incorporation was similar in wild-type and c-Abl-null B cells in liquid culture assays (Fig. 1B). Similar results were observed in B cells from abl-2 mice crossed with the outbred strain CD-1 (data not shown). These data are in agreement with in Fig. 3, the c-Abl SH2 domain interacted with a tyrosine-phosphorylated and interacts with SH2-containing proteins following activation of the BCR, Ramos cells were stimulated with anti-human IgM F(ab’)2, Ab fragments and then fractionated into soluble cytosol, insoluble membrane/cytoskeleton, and nuclear fractions. As depicted in Fig. 2 (top), c-Abl kinase activity was elevated in the soluble fraction following stimulation of the BCR (1.7 ± 0.2-fold after 5 min, n = 3). This increase in kinase activity was paralleled by an increase in c-Abl protein levels in the cytosol (Fig. 2, middle). No alteration of c-Abl protein levels or kinase activity was apparent in either the Triton-insoluble membrane/cytoskeletal or nuclear fractions (data not shown). The purity of the fractionation was verified by Western blotting for proteins specific to each fraction (data not shown). Similar results were obtained in another cell line, Raji (data not shown). LPS treatment of B cells in liquid culture did not result in an alteration of c-Abl protein levels or kinase activity (data not shown). These data demonstrate that c-Abl is regulated following engagement of the BCR.

c-Abl interacts with CD19

Because c-Abl is regulated by activation of the BCR, it was of interest to identify signaling molecules that associate with or are regulated by c-Abl. The BCR coreceptor CD19 is highly tyrosine phosphorylated and interacts with SH2-containing proteins following B cell activation (12, 13). Two phosphotyrosines in CD19 have been identified as potential binding sites for the Abl SH2 domain (18). To determine whether the c-Abl SH2 domain interacts with CD19, lysates from unstimulated or stimulated Ramos cells were incubated with a GST-ABL SH2 domain fusion protein. As shown in Fig. 3A, the c-Abl SH2 domain interacted with a tyrosine-phosphorylated protein of ~95 kDa only after activation of the BCR. No interaction with the phosphoprotein was observed with GST

FIGURE 1. Anti-IgM-induced proliferation is reduced in B cells lacking c-Abl. Splenic B lymphocytes were isolated from wild-type (+/+ or ab/2) knockout (−/−) mice (>90% pure as analyzed by FACS analysis) and treated with increasing amounts of goat anti-mouse IgM F(ab’)2, Ab fragments (A) or LPS (B) for 72 h. Results represent the mean incorporation of [3H]thymidine ± SD of triplicate samples and are representative of four (A) and two (B) experiments.
alone. This protein was not detected when lysates were precleared with an anti-CD19 Ab, but remained when lysates were not precleared or were precleared with an irrelevant Ab. These results suggest that the tyrosine-phosphorylated protein is CD19 or is in a complex with CD19. Western blotting with antisera against CD19 demonstrated that CD19 was present in the GST-Abl SH2 pull-down only after stimulation (Fig. 3B). Direct interaction of the c-Abl SH2 domain with CD19 was observed in Far Western blots (data not shown). These results demonstrate that tyrosine-phosphorylated CD19 can associate with the c-Abl SH2 domain following B cell activation.

We next examined whether CD19 could interact with c-Abl in vivo. We first expressed CD19 and c-Abl alone and together in Bosc23 cells and examined whether the proteins coimmunoprecipitated from cell lysates. c-Abl was present in CD19 immunoprecipitates (Fig. 4A) and, conversely, CD19 was present in c-Abl immunoprecipitates (data not shown) only when the proteins were coexpressed. Western blotting of whole cell lysates (WCL) and of the immunoprecipitates with Abs to c-Abl and CD19 verified the expression of each protein (Fig. 4A). Similar results were obtained with different Abs to c-Abl and CD19, raised against distinct epitopes in the two proteins (data not shown). Neither protein was observed when control Abs were used.

We further examined the association of endogenous c-Abl and CD19 in Ramos cells. Endogenous c-Abl and CD19 proteins coimmunoprecipitated as a constitutive complex (Fig. 4B). Surprisingly, the complex was not quantitatively altered following BCR stimulation (Fig. 4B). Together, these results demonstrate that c-Abl and CD19 form a constitutive molecular complex in vivo in unstimulated Ramos cells, and following BCR stimulation the interaction is mediated in part by the c-Abl SH2 domain with tyrosine-phosphorylated CD19.

**CD19 is a substrate of the c-Abl tyrosine kinase**

CD19 can interact with the SH2 domains of multiple proteins (12), including that of c-Abl (Fig. 3). However, little is known regarding the kinases that directly phosphorylate CD19. To determine whether CD19 is a substrate of the c-Abl kinase, we first examined whether CD19 was phosphorylated in the presence of c-Abl in vivo. As shown in Fig. 5, immunoprecipitated CD19 was tyrosine phosphorylated only when CD19 was coexpressed with wild-type c-Abl. No phosphorylation was observed in cells lacking c-Abl or CD19. Furthermore, this phosphorylation was dependent upon the

**FIGURE 3.** The c-Abl SH2 domain interacts with CD19 following B cell activation. A. Lysates from Ramos cells stimulated with anti-IgM were precleared alone, with anti-CD19 Abs, or an anti-protein kinase C β1 Ab (negative control). Ab complexes were removed by binding to protein A- or G-Sepharose, and the remaining supernatant was incubated with GST or GST-Abl SH2. Bound proteins were analyzed for phosphotyrosine by Western blotting. B. Ramos cells were stimulated for the indicated times. Cell lysates were incubated with a GST-Abl SH2 fusion protein. Proteins bound to GST-Abl SH2 (left) or WCL (right) were analyzed by Western blotting for CD19.

**FIGURE 4.** CD19 interacts with c-Abl in vivo. A, Bosc23 cells were transfected with plasmids encoding c-Abl and/or full-length CD19 as indicated. Cell lysates were prepared and proteins were immunoprecipitated with the anti-CD19 antiserum 5381, or control preimmune serum (PI). Immunoprecipitates were analyzed by Western blotting with the indicated Abs. Blots were stripped and reprobed for the indicated immunoprecipitated proteins. Expression of the proteins in WCL is shown on the right. B, Lysates from anti-IgM-stimulated Ramos cells were immunoprecipitated with anti-CD19 serum or preimmune (PI) serum. Immunoprecipitates and WCL were analyzed by Western blotting for c-Abl (top). Blots were stripped and reprobed with anti-CD19 antiserum to show equal immunoprecipitation of CD19 (middle). Blots were stripped again and reprobed for phosphotyrosine before and after B cell stimulation (bottom).

**FIGURE 5.** CD19 is phosphorylated in vivo when coexpressed with c-Abl. Bosc23 cells were transfected with plasmids encoding c-Abl wild type (wt) or kinase inactive (kr) and/or full-length CD19 as indicated. Cell lysates were prepared and proteins were immunoprecipitated with the anti-CD19 antiserum 5381 or preimmune (PI) serum. Immunoprecipitates were analyzed by Western blotting with the indicated Abs. WCL were blotted for c-Abl protein expression.
presence of c-Abl kinase activity, as coexpression of a kinase inactive c-Abl (K290R) did not result in phosphorylation of CD19.

It is noteworthy that the migration of the CD19 protein was altered when coexpressed with c-Abl wild type (Figs. 4A and 5). Overexpressed CD19 in the absence of c-Abl migrated as a doublet of ∼80–95 kDa. When coexpressed with wild-type c-Abl, the relative amounts of the CD19 doublet were altered such that the lower band was more prominent (Figs. 4A and 5). The levels of the 95-kDa form of CD19, which is heavily tyrosine phosphorylated, were reduced in the presence of kinase-active c-Abl (compare Fig. 5 upper panel to middle panel). Furthermore, in several experiments expression of c-Abl wild type induced the accumulation of an even faster migrating form of CD19 of ∼75 kDa (Fig. 4A). This species may represent a cleavage product of CD19, or CD19 molecules that are differentially posttranslationally modified, as the predicted molecular mass of unmodified CD19 is ∼65 kDa. These results may also suggest that the hyperphosphorylated form of CD19 is targeted for internalization and/or degradation in the presence of kinase-active c-Abl.

We next examined whether c-Abl could directly phosphorylate CD19 in vitro. As shown in Fig. 6A, c-Abl produced in 293T cells phosphorylated the GST-CD19 tail encompassing amino acids 362–540, as well as the known c-Abl substrate GST-Crk. No phosphorylation of GST alone was observed (data not shown). Furthermore, only wild-type c-Abl and not a kinase-inactive mutant (K290R) could phosphorylate CD19, ruling out the presence of a coprecipitating kinase (Fig. 6A). These results demonstrate that CD19 is a substrate of c-Abl in vitro.

Because the GST-CD19 fragment used here contains seven potential sites of tyrosine phosphorylation, the phosphorylation of CD19 by c-Abl was further analyzed by mass spectrometry. This analysis revealed that CD19 is phosphorylated by c-Abl at only one site (Fig. 6B). Unphosphorylated GST-CD19 tail (top) had a molecular mass of ∼45,540 Da, whereas that of GST-CD19 phosphorylated by c-Abl was ∼45,629 Da. This shift in molecular mass reflects the additional mass of one phosphate group. The minor peaks to the left of the major peaks represent CD19 minus one methionine residue in the unphosphorylated and phosphorylated states. These results suggest that only one site on CD19 is phosphorylated by c-Abl. However, the data can be interpreted to mean that c-Abl phosphorylates CD19 at one specific site, or that c-Abl phosphorylates one site on any single CD19 molecule, but that multiple individual tyrosines may be targeted. To determine the identity of the tyrosine(s) phosphorylated by c-Abl, phosphopeptide sequence analysis was performed. The results revealed that only tyrosine (Y)490 of CD19 was phosphorylated by c-Abl. This site of phosphorylation of CD19 by c-Abl is the same as that predicted by phage display analysis (19).

c-Abl and CD19 colocalize in detergent-insoluble lipid rafts

To further examine whether endogenous c-Abl and CD19 colocalize in B cells, we investigated whether both proteins are present in lipid rafts isolated from Ramos cells. Lipid rafts are sphingolipid/cholesterol-enriched membrane microdomains that have been proposed to function as platforms for signal transduction events and are rich in signaling molecules including the subunits of the BCR, the Lyn tyrosine kinase, CD20, CD40, and phospholipase C (PLC)-γ2 in B cells (28–31). The data in Fig. 7 (top and middle) demonstrate that c-Abl and CD19 are present in the detergent-insoluble lipid rafts in Ramos cells. Both proteins are constitutively associated with the membrane rafts, and their presence in lipid rafts is not altered following BCR stimulation. We show that the Lyn tyrosine kinase is also present in lipid rafts (Fig. 7, bottom), as previously reported (28, 29). The concentration of the c-Abl tyrosine kinase appeared to be equivalent between the soluble fraction and the lipid rafts under the conditions used here. In contrast, there was less CD19 present in the lipid rafts than in the soluble fraction. Interestingly, the relative level of tyrosine-phosphorylated CD19 in the lipid rafts was elevated relative to that found in the soluble fraction (Fig. 8). Thus, a higher proportion of CD19 molecules, or available tyrosines on CD19, are phosphorylated in this membrane microdomain following BCR stimulation. Overall, these results demonstrate that c-Abl and CD19 colocalize in the detergent-insoluble lipid raft microsignaling domain. This

FIGURE 6. CD19 is an in vitro substrate for the c-Abl kinase. A, Anti-Abl immunoprecipitates from lysates of 293T cells transfected with plasmids encoding either wild type (wt) or kinase inactive (kr) c-Abl were incubated with GST-CD19 tail in the presence of [γ-32P]ATP. The Ab substrate GST-Crk was used as a positive control. Migrations of the GST fusion proteins are indicated on the left. B, Mass spectrometric tracings of unphosphorylated CD19 (top) or CD19 phosphorylated by c-Abl (bottom) are shown. The site of phosphorylation mapped by peptide sequencing is indicated (bottom).

FIGURE 7. c-Abl and CD19 are present in lipid rafts. Ramos B cells were stimulated as above and lipid rafts were isolated by ultracentrifugation through a discontinuous sucrose density gradient as described in Materials and Methods. The presence of c-Abl (top) and CD19 (middle) in the soluble and raft fractions (5 μg) was analyzed by Western blotting with the indicated Abs. As a positive control for lipid raft isolation, the samples were Western blotted for the Lyn tyrosine kinase (bottom).
colocalization may have a functional significance given the high fraction of tyrosine-phosphorylated CD19 present in this fraction.

Discussion

In this report we have shown that the c-Abl tyrosine kinase is required for maximal proliferation of B cells. A role for c-Abl in BCR signaling may be explained by our finding that c-Abl interacts with and phosphorylates CD19, a scaffold protein that recruits signaling components following BCR engagement. A reduction of BCR-induced proliferation is observed in B cells from mice that are c-Abl null (Fig. 1) or that express a truncated c-Abl lacking the carboxyl terminus (7). It is noteworthy that mice with a targeted deletion of CD19 exhibit a similar reduction of BCR-induced proliferation (32). This suggests that c-Abl and CD19 may act in a similar pathway leading to BCR-mediated growth regulation of B cells. The reduced proliferation of B cells lacking c-Abl may be the result of increased apoptosis. In this regard, embryos from mice that are doubly deficient for c-abl and the c-abl-related kinase arg, and B cell lines derived from the mutant abl<sup>−/−</sup> mice have an increased susceptibility to apoptosis (8, 33), whereas cells expressing the activated Abl oncoproteins are resistant to apoptosis induced by a variety of stimuli (reviewed in Ref. 34). Alternatively, the reduced BCR-mediated proliferation in c-Abl- and CD19-deficient B cells may be due to a signaling defect in a proliferative pathway(s) modulated by these two molecules. In this regard, CD19 may modulate signaling thresholds and function to enhance the proliferative response to external signals (12). Similar roles may be ascribed to c-Abl. Thus, in the absence of c-Abl, reduced phosphorylation of CD19 and other c-Abl substrates may impair recruitment of signaling components to the BCR complex and reduce the proliferative or survival potential of B cells.

The link between c-Abl and CD19 is further strengthened by our observation that the subcellular distribution of c-Abl is regulated following engagement of the BCR (Fig. 2). The c-Abl protein levels and kinase activity increase in the cytosol following stimulation of the BCR in two independent B cell lines. One possible explanation for these findings is described below. We have shown that c-Abl and CD19 form a constitutive complex in B cells that does not appear to be increased following stimulation of the BCR (Fig. 4B). This observation is similar to that reported for the association of CD19 and the Lyn tyrosine kinase. Lyn and CD19 form a constitutive complex in primary B cells, and BCR stimulation increases Lyn association with CD19 by only ~2-fold (17). Tyrosine phosphorylation and activation of the Lyn tyrosine kinase have been shown to produce a significant decrease in the binding capacity of the Lyn SH2 domain for phosphotyrosine-containing targets (35). Thus, the reduced affinity of the activated Lyn kinase for tyrosine-phosphorylated CD19 may result in the release of Lyn from the CD19 complex into the cytosol. Similarly, following BCR engagement, a pool of c-Abl in the vicinity of the BCR may be autoprophosphorylated or phosphorylated by Src family kinases (4), and the tyrosine-phosphorylated c-Abl may then be released into the cytosol as a consequence of the decreased binding affinity between activated c-Abl and CD19. This possibility is consistent with our observation of increased c-Abl kinase in the cytosol following BCR stimulation (Fig. 2).

We have also shown here for the first time that the BCR coreceptor CD19 is a substrate for the c-Abl kinase in vitro and in vivo. Although tyrosine phosphorylation of CD19 downstream of numerous signals has been reported (12–14), the kinases that mediate the phosphorylation of CD19 downstream of these signals have not been identified. Recently, the Lyn tyrosine kinase was shown to phosphorylate CD19 in vitro and in vivo (11, 17). Significantly, CD19 was not tyrosine phosphorylated in resting or activated B cells isolated from Lyn<sup>−/−</sup> mice following BCR ligation. In contrast, CD19 was tyrosine phosphorylated in B cells from wild-type mice, and this phosphorylation was elevated following BCR stimulation (17). Here we show that c-Abl phosphorylates CD19 at a specific tyrosine, Y490. Specific tyrosine-phosphorylated sites on CD19 have been shown to interact with various SH2-containing proteins (12). However, Y490 has not been reported or predicted to interact with known SH2-domain containing proteins (17), although it is in the context of a putative SH2-binding motif (YXXP, where X is any amino acid; Ref. 18). Interestingly, Y490 lies between two binding sites for the SH2 domains of the p85 subunit of phosphatidylinositol 3-kinase and the Src family tyrosine kinases (Y482 and Y513) (12, 17). Thus, it is possible that phosphorylation of the c-Abl site may modulate binding of other proteins to these adjacent phosphotyrosines. In this case, phosphorylation of Y490 would serve to modulate CD19 complex interactions rather than directly recruit complex components. Alternatively, phosphorylation of Y490 by c-Abl may recruit an SH2-containing protein that participates in CD19 signaling. Recruitment of such a protein may affect the binding of phosphatidylinositol 3-kinase or Src kinases to the adjacent sites or may regulate other BCR-mediated signaling pathways.

In addition to its ability to phosphorylate CD19, we demonstrate that c-Abl can associate with this BCR coreceptor. The SH2 domain of c-Abl associates with CD19 only after stimulation of B cells, suggesting that tyrosine phosphorylation of CD19 is required for this interaction. However, coimmunoprecipitation of endogenous CD19/c-Abl complexes from Ramos B cells revealed that these proteins are constitutively associated (Fig. 4B). Furthermore, the coimmunoprecipitated c-Abl/CD19 complex did not appear to be increased after BCR stimulation. In agreement with these data, we have also observed that c-Abl kinase activity is not required for this interaction (data not shown). These data suggest that these proteins associate in the absence of detectable CD19 tyrosine phosphorylation (Figs. 4A and 5, and data not shown) or B cell activation (Fig. 4B). Similarly, as described above, the Lyn tyrosine kinase forms a constitutive complex with CD19 that is only slightly elevated following BCR stimulation (17). Activation of

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**FIGURE 8.** CD19 is highly phosphorylated within lipid rafts. Lipid rafts were isolated from Ramos B cells as above and immunoprecipitated with normal rabbit serum (NRS) or the anti-CD19 anti-serum 5381. The soluble fraction was immunoprecipitated for comparison. Immunoprecipitates were analyzed by Western blotting for phosphotyrosine (top). The blot was stripped and reprobed with anti-CD19 antisera (bottom).
c-Abl and/or hyperphosphorylation of CD19 may result in a qualitative change in the complex that causes an unstable SH2-mediated interaction between c-Abl and CD19 such that an increase in stable complexes containing CD19 and c-Abl are not observed following BCR stimulation. Furthermore, it has been documented that substrate/kinase interactions are difficult to isolate, such as that described for the interaction of the Src tyrosine kinase and its substrate, the lipid raft-associated phosphoprotein associated with glycosphingolipid-enriched microdomains/CSK binding protein (36, 37).

Results from phage display analysis predict that the c-Abl SH2 domain may bind to phosphorylated Y391 and Y421 of CD19 (18). These phosphorylated tyrosines of CD19 are the sites of interaction with the SH2 domain of the Vav protein, and tyrosine to phenylalanine (F) mutation (Y391F, Y421F) of these sites reduces this interaction and alters signal transduction downstream of the BCR and CD19 (38–40). However, not all the effects of the tyrosine to phenylalanine mutations can be accounted for by the loss of Vav binding to CD19. The CD19 Y391F and Y421F mutants exhibit reduced Ca2+ flux and activation of Erk following stimulation of the BCR alone or costimulation of both the BCR and CD19 (38–40). Significantly, the observed decrease in Erk activation cannot be due to loss of Vav binding, as Erk activation is unaltered in BCR-stimulated B cells from Vav-deficient mice (38, 41). This suggests that SH2-containing proteins other than Vav may bind to these sites to regulate distinct signaling pathways. Recently, PLC-γ-2 has also been shown to interact with these residues and may be important for signaling downstream of CD19 (40). Our results suggest that c-Abl may be yet a third protein that interacts with these sites on CD19. Thus, the c-Abl kinase may alter protein associations with CD19, as well as recruit and phosphorylate substrates to transduce signals downstream of CD19.

In addition to complex formation between c-Abl and CD19, we have demonstrated for the first time that c-Abl and CD19 colocalize as components of the detergent-insoluble lipid raft microsizing domains (Fig. 7). These microsizing domains are dynamic sites of signal transduction, and accumulating evidence suggests that lipid rafts play a crucial role in immune receptor signal initiation and propagation (42). We and others have found that c-Abl, CD19, and the Src tyrosine kinase family member Lyn are constitutively associated with lipid rafts in B cells (Fig. 7) (28, 29). Furthermore, proteins including the BCR Ig and Ig α/β subunits and PLC-γ-2 have been shown to translocate into lipid rafts following BCR stimulation (28, 29). Both CD19 (Fig. 8) and Lyn (29) are hyperphosphorylated in lipid rafts following B cell stimulation, suggesting that these proteins are important mediators of signaling within lipid rafts. Interestingly, in resting T cells the linker for activation of T cells (LAT) adaptor protein and the Src family member Lck have been identified as constitutive components of lipid rafts (43). The localization of both the adaptor protein LAT and the tyrosine kinase Lck to membrane rafts is required for efficient signal transduction downstream of the TCR. Lck and LAT are believed to function as integral mediators of early signal transduction by respectively phosphorylating and recruiting numerous signaling proteins to these microsizing domains (reviewed in Ref. 43). A similar scenario may be envisioned in B cells where the constitutive association of Lyn, c-Abl, and CD19 in lipid rafts may play a role in the transduction of early signaling events downstream of the BCR.

In summary, the data presented here suggest a model for signaling events downstream of the BCR. Crosslinking of the BCR results in its translocation to rafts where Lyn, c-Abl, and CD19 are preassembled. Lyn becomes activated and phosphorylates the BCR CD79α subunit, the CD19 coreceptor, and other proteins. Phosphorylated CD19 then acts as a scaffolding protein to bind SH2 domains, such that of the c-Abl tyrosine kinase. Once recruited to CD19, c-Abl may be activated via the CD19/Src amplification loop (11, 17), as we have previously shown that Src kinases phosphorylate and activate c-Abl (4). Tyrosine phosphorylation of CD19 leads to the recruitment of additional signaling molecules (12, 17) that may serve as substrates for the c-Abl tyrosine kinase. These signaling molecules may together contribute to the regulation of survival and/or proliferative pathways downstream of the BCR. The absence of c-Abl in B cells may affect the formation of a fully functional CD19 signaling complex, leading to the reduced proliferation observed in c-Abl-null B cells following activation of the BCR.

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