CD19 Amplification of B Lymphocyte Ca\(^{2+}\) Responses

A ROLE FOR Lyn SEQUESTRATION IN EXTINGUISHING NEGATIVE REGULATION*

Manabu Fujimoto‡§, Jonathan C. Poe‡, Minoru Hasegawa, and Thomas F. Tedder¶

From the Department of Immunology, Duke University Medical Center, Durham, North Carolina 27710

B lymphocyte antigen receptor (BCR) signals are regulated by CD19, with BCR-induced intracellular calcium ([Ca\(^{2+}\)]) responses enhanced by CD19 co-ligation. In this study, CD19 engagement using a dimeric anti-CD19 antibody induced [Ca\(^{2+}\)], mobilization and significantly enhanced BCR-induced [Ca\(^{2+}\)], responses without a requirement for CD19/BCR co-ligation. Although simultaneous CD19 and BCR engagement significantly enhanced CD19/Lyn complex formation and [Ca\(^{2+}\)], responses, downstream tyrosine phosphorylation of CD22 and multiple other cellular proteins was inhibited, as was SHP1 recruitment to phosphorylated CD22. CD19 overexpression also enhanced BCR-induced [Ca\(^{2+}\)] responses, but down-regulated tyrosine phosphorylation of CD22 and multiple other cellular proteins following BCR ligation. Because CD19 and Lyn expression are genetically titrated in B cells, CD19 engagement may augment BCR-induced [Ca\(^{2+}\)], responses by sequestering the available pool of functional Lyn away from downstream effector pathways as such as CD22. Consistent with this, simultaneous CD19 engagement did not further enhance the BCR-induced [Ca\(^{2+}\)], responses of Lyn- or CD22-deficient B cells. Thus, CD19 recruitment of Lyn may preferentially activate selective signaling pathways downstream of the CD19/Lyn complex to the exclusion of other downstream regulatory and effector pathways. Other receptors may also utilize a similar strategy to regulate kinase availability and downstream intermolecular signaling.

B lymphocyte development and function are regulated by signals transduced through the B cell antigen receptor (BCR) and cell surface regulatory molecules including CD19 (1–3). CD19 is a member of the immunoglobulin superfamily expressed exclusively on B cells and follicular dendritic cells (4). CD19 has a ~240-amino acid cytoplasmic domain that is critical for CD19 signaling (4–6), and CD19-deficient (CD19\(^{-/-}\)) mice are hyporesponsive to a variety of transmembrane signals (7–9). Specifically, CD19 functions as a specialized adapter protein for the amplification of Src family protein-tyrosine kinase (PTK) activity and as an interaction molecule for multiple signaling pathways crucial for modulating intrinsic and antigen receptor-induced signals (10–12). The cytoplasmic domains of human CD19 and mouse CD19 are highly homologous (13). In fact, human CD19 can replace mouse CD19 function when expressed at the appropriate site density in CD19\(^{-/-}\) mice (14). Overexpression of CD19 in transgenic mice expressing a human CD19 transgene (CD19GTG mice) renders B cells hyperresponsive to transmembrane signals (7, 15, and 16). Thus, CD19 is generally considered a positive regulator of B cell function because it amplifies Src family PTK activation, mitogen-activated protein kinase (MAPK) activation, and proliferation (11, 17, and 18). Moreover, CD19 and BCR co-ligation greatly augments BCR-induced ([Ca\(^{2+}\)]) responses (19) and dramatically lowers the threshold for B cell activation in vitro (17, 20).

Although CD19 and BCR co-ligation can significantly enhance [Ca\(^{2+}\)], responses (17, 19), it is unclear whether CD19 ligation can augment BCR-induced [Ca\(^{2+}\)], responses independent of BCR and CD19 coengagement. Moreover, CD19 ligation can also inhibit BCR-induced [Ca\(^{2+}\)], responses and suppress B cell proliferation (21–25). Although the molecular mechanisms through which CD19 augments or inhibits BCR-induced [Ca\(^{2+}\)], responses are unknown, the above studies suggest that the extent, valency, or timing of CD19 ligation may be crucial to establishing a balance between positive and negative regulatory signals and signals transduced via the BCR. To address these issues, we have generated a dimeric IgA anti-mouse CD19 monoclonal antibody (16) that allows assessment of the functional consequences of CD19 cross-linking independent of BCR ligation. Independent CD19 and BCR engagement was found to augment BCR-induced [Ca\(^{2+}\)], responses and qualitatively regulate BCR signaling through potentially novel mechanisms depending on the extent or valency of CD19 ligation.

EXPERIMENTAL PROCEDURES

Mice—CD22-deficient (CD22\(^{-/-}\); 129 \(\times\) C57Bl/6), CD19TG (C57Bl/6) and Lyn\(^{-/-}\) (129 \(\times\) C57Bl/6, provided by Dr. C. Lowell, University of California, San Francisco) mice were 2–3 months of age and were housed in a specific pathogen-free barrier facility as described (7, 26, and 27). Wild type littermates from heterozygous breedings were used as controls, respectively. All procedures were approved by the Duke University Animal Care and Use Committee.

Antibodies—The mouse anti-mouse CD19 mAb (MB19-1, IgA) used in these studies was generated as described (16) and purified by affinity chromatography using DEAE-Sepharose beads (Amersham Pharmacia Biotech). Other mAbs used included rat anti-mouse CD19 (1D3, IgG2a; BD Pharmingen, San Diego, CA); anti-CD22 (Cy34, TIB163, American Type Culture Collection, Manassas, VA); anti-CD22 cytoktoplastic domain.
main (MB22-1, Ref. 28); anti-B220 (RA3-6B2; BD PharMingen); and horseradish peroxidase (HRP)-conjugated anti-phosphotyrosine antibodies (4G10, Upstate Biotechnology, Lake Placid, NY; PY99, Santa Cruz Biotechnology, Santa Cruz, CA). Antisera used included anti-Lyn, anti-Syk, anti-phospholipase C-γ2 (PLC-γ2), anti-extracellular signal-regulated kinase (ERK) 2, and anti-dually phosphorylated active ERK (all from Santa Cruz Biotechnology); anti-Src homology-2-containing protein-tyrosine phosphatase 1 (SHP1; Upstate Biotechnology); rabbit anti-CD19 cytoplasmic domain antisera (provided by Dr. M. Grove, Duke University); and anti-CD79a antisera (provided by Dr. L. Matsuuchi, University of British Columbia, Vancouver, Canada).

Measurement of [Ca²⁺]i Responses—Spleen cells (10⁷/ml) in RPMI 1640 medium containing 5% fetal calf serum at 37°C were loaded with 1 μM indo-1-AM ester (Molecular Probes, Eugene, OR) at 37°C for 30 min. The cells were washed and stained with FITC-conjugated anti-B220 antibody for 15 min at room temperature and washed. The ratio of fluorescence (525/405 nm) of B220 plus rabbit antiserum overnight at 4°C. Immunoprecipitated proteins were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto membranes for immunoblotting. The blots were blocked with HRP-conjugated antibody to detect protein-tyrosine phosphorylation or were incubated with antibodies specific for Lyn, Syk, anti-phospholipase C-2 (ALC-2; Transduction, San Diego, CA), and anti-phospho-CD79 γ (Upstate Biotechnology) and 10 μM (Sigma) were added 10 min prior to fluorescence ratio measurements of cells (2 × 10⁶/ml). Results were plotted as the fluorescence ratio at 20-s intervals with the background subtracted. Increased fluorescence ratios indicate increases in [Ca²⁺].

**B Cell Activation, Immunoprecipitations, and Western Blot Analysis—**Spleen B cells were resuspended (2 × 10⁷/ml) into RPMI 1640 medium containing 5% fetal calf serum at 37°C. Cells were stimulated with goat anti-mouse IgM antibody fragment (F(ab′)2) and MB19-1 antibody and subsequently lysed as described (11). Protein concentrations were determined by light absorbance at 280 nm. For immunoprecipitations, the cell lysates were precleared twice by incubation with appropriate control antibodies plus protein G-Sepharose beads (Amersham Pharmacia Biotech), followed by incubation with protein G beads plus rabbit antisera overnight at 4°C. Immunoprecipitated proteins were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto membranes for immunoblotting. The blots were blocked with HRP-conjugated antibody to detect protein-tyrosine phosphorylation or were incubated with antibodies specific for proteins of interest, followed by incubation with HRP-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). The blots were developed using an enhanced chemiluminescence kit (Pierce). The blots were stripped and re-probed with antibodies specific for the protein of interest to verify equivalent amounts of protein in each lane.

**In Vitro Lyn Kinase Assays—**Spleen B cell lysates were precleared and incubated with anti-Lyn antisera and protein A-Sepharose beads for 3 h at 4°C. The beads were subsequently washed four times in lysis buffer and twice in reaction buffer (50 mM Tris-Cl, pH 7.4, 10 mM MgCl₂, 0.1 mM sodium orthovanadate, 1 mM diithiothreitol). The beads in 50 μl of reaction buffer were incubated with 10 μg of cdc25-20NH₂ peptide (Upstate Biotechnology) and 10 μCi of [γ-³²P]ATP for 2 min at 25°C. The reactions were terminated by adding 40% trichloroacetic acid, and spotted onto p81 phosphocellulose paper. The phosphocellulose paper was washed five times with 0.5% phosphoric acid, and once with acetic acid. Radioactivity was quantified by scintillation counting.

**RESULTS**

**Independent CD19 and BCR Ligation Positively Regulates [Ca²⁺]i Responses—**CD19 engagement on mouse splenic B cells using a purified, dimeric mouse IgA anti-CD19 mAb induced a dose-dependent rise in [Ca²⁺], without the need for a cross-linking secondary antibody. CD19 engagement using this antibody at 5–20 μg/ml induced significant [Ca²⁺]i responses (Fig. 1). Maximal [Ca²⁺]i responses induced by anti-CD19 antibody at 5–20 μg/ml were only slightly faster than higher. A control mouse IgA monoclonal antibody did not induce detectable [Ca²⁺]i responses when used at any concentration (5–80 μg/ml, data not shown). Thus, CD19 signaling complex engagement was sufficient to induce significant [Ca²⁺]i responses.

Compared with the [Ca²⁺]i responses induced following CD19 engagement using the dimeric IgA antibody (5–40 μg/ml), BCR-induced [Ca²⁺]i responses were larger, with an initial rapid phase followed by a sustained increase in [Ca²⁺]i (Fig. 2A).

**Mechanisms for Augmented [Ca²⁺]i Responses Following CD19/BCR Ligation—**Alterations in B cell protein-tyrosine phosphorylation were assessed to determine the molecular basis for augmented [Ca²⁺]i responses following simultaneous but independent CD19 and BCR ligation. BCR engagement induced the tyrosine phosphorylation of multiple cellular proteins, whereas CD19 ligation-induced changes were modest (Fig. 3A). Surprisingly, overall protein phosphorylation induced by simultaneous BCR (40 μg/ml antibody) and CD19 (10 μg/ml antibody) ligation was significantly less intense than with BCR ligation alone (Fig. 3A), independent of the time point examined (data not shown). By contrast, CD19 phosphorylation was significantly augmented by simultaneous CD19 and BCR ligation when compared with BCR or CD19 ligation alone (Fig. 3B) as described previously (30). Because Lyn is the primary kinase that phosphorylates CD19, and CD19 amplifies Lyn kinase activity following BCR ligation (11, 12), more Lyn was associated with CD19 following simultaneous CD19 and BCR ligation than with BCR or CD19 ligation alone (Fig. 3B).

Thus, simultaneous CD19 and BCR ligation augments [Ca²⁺]i responses while reducing overall protein-tyrosine phosphorylation. In contrast, increased CD19 phosphorylation and enhanced Lyn recruitment to CD19.

Despite decreased overall protein-tyrosine phosphorylation in B cells following simultaneous BCR and CD19 ligation (Fig. 3A), this did not down-regulate Lyn phosphorylation or kinase activity (Fig. 3C). Simultaneous BCR and CD19 ligation did not alter CD79a phosphorylation (Fig. 3D). Yet, 20–22% of levels induced by IgM ligation alone, n = 3). Syk phosphorylation was slightly decreased following simultaneous BCR and CD19 liga-
Role of PI 3-Kinase in CD19/BCR-induced \([\text{Ca}^{2+}]\), Responses—Effect molecules participating in BCR-induced \([\text{Ca}^{2+}]\) responses in addition to Lyn, CD79, Syk and PLC-\(\gamma\) include PI 3-kinase, which is recruited to the cell surface by multiple tyrosine-phosphorylated proteins, including CD19 (31 and 32). Therefore, PI 3-kinase-specific inhibitors, wortmannin and LY294002, were used to determine whether increased PI 3-kinase binding to CD19 and other cell surface molecules selectively augmented \([\text{Ca}^{2+}]\), responses following simultaneous BCR and CD19 ligation. Both inhibitors blocked all kinase activity during in vitro assays with PI 3-kinase immunoprecipitated from B cells (data not shown) when used at concentrations previously shown to inhibit PI 3-kinase activity in B cells under similar, if not identical, conditions (33–35). CD19-induced \([\text{Ca}^{2+}]\), responses were nearly eliminated by both PI 3-kinase inhibitors (Fig. 4, data not shown). By contrast, simultaneous BCR and CD19 ligation induced exaggerated \([\text{Ca}^{2+}]\), responses in the presence of inhibitors, when compared with BCR ligation alone in the presence of inhibitors (Fig. 4, data not shown). Higher concentrations of both inhibitors generated similar results (data not shown). Thus, even in the absence of PI 3-kinase activity, simultaneous CD19/BCR ligation induced augmented \([\text{Ca}^{2+}]\), responses. Therefore, signaling molecules in addition to PI 3-kinase are likely to contribute to the augmented \([\text{Ca}^{2+}]\), responses induced by simultaneous CD19/BCR ligation.

Simultaneous CD19/BCR Ligation Inhibits CD22 Phosphorylation—That protein-tyrosine phosphorylation induced by simultaneous BCR (40 \(\mu\)g/ml antibody) and CD19 (10 \(\mu\)g/ml antibody fragments and/or anti-CD19 antibody (MB19-1) were used to determine whether increased PI 3-kinase, which is recruited to the cell surface by multiple signaling molecules, including CD19 (31 and 32), BCR and simultaneous CD19 and BCR ligation. Both inhibitors blocked all kinase activity during in vitro assays with PI 3-kinase immunoprecipitated from B cells (data not shown) when used at concentrations previously shown to inhibit PI 3-kinase activity in B cells under similar, if not identical, conditions (33–35). CD19-induced \([\text{Ca}^{2+}]\), responses were nearly eliminated by both PI 3-kinase inhibitors (Fig. 4, data not shown). By contrast, simultaneous BCR and CD19 ligation induced exaggerated \([\text{Ca}^{2+}]\), responses in the presence of inhibitors, when compared with BCR ligation alone in the presence of inhibitors (Fig. 4, data not shown). Higher concentrations of both inhibitors generated similar results (data not shown). Thus, even in the absence of PI 3-kinase activity, simultaneous CD19/BCR ligation induced augmented \([\text{Ca}^{2+}]\), responses. Therefore, signaling molecules in addition to PI 3-kinase are likely to contribute to the augmented \([\text{Ca}^{2+}]\), responses induced by simultaneous CD19/BCR ligation.

Simultaneous CD19/BCR Ligation Inhibits CD22 Phosphorylation—That protein-tyrosine phosphorylation induced by simultaneous BCR (40 \(\mu\)g/ml antibody) and CD19 (10 \(\mu\)g/ml antibody fragments and/or anti-CD19 antibody (MB19-1) were used to determine whether increased PI 3-kinase, which is recruited to the cell surface by multiple signaling molecules, including CD19 (31 and 32), BCR and simultaneous CD19 and BCR ligation. Both inhibitors blocked all kinase activity during in vitro assays with PI 3-kinase immunoprecipitated from B cells (data not shown) when used at concentrations previously shown to inhibit PI 3-kinase activity in B cells under similar, if not identical, conditions (33–35). CD19-induced \([\text{Ca}^{2+}]\), responses were nearly eliminated by both PI 3-kinase inhibitors (Fig. 4, data not shown). By contrast, simultaneous BCR and CD19 ligation induced exaggerated \([\text{Ca}^{2+}]\), responses in the presence of inhibitors, when compared with BCR ligation alone in the presence of inhibitors (Fig. 4, data not shown). Higher concentrations of both inhibitors generated similar results (data not shown). Thus, even in the absence of PI 3-kinase activity, simultaneous CD19/BCR ligation induced augmented \([\text{Ca}^{2+}]\), responses. Therefore, signaling molecules in addition to PI 3-kinase are likely to contribute to the augmented \([\text{Ca}^{2+}]\), responses induced by simultaneous CD19/BCR ligation.
CD19 Ligation Uncouples CD22 Negative Regulation

Fig. 4. Calcium responses induced by BCR and/or CD19 ligation in the presence of PI 3-kinase-specific inhibitors. Calcium responses in wild type B cells pretreated with LY294002 for 10 min before IgM ligation with anti-IgM (40 μg/ml), anti-CD19 antibody (40 μg/ml), or anti-IgM plus anti-CD19 antibody (10 μg/ml). Spleen cells were loaded with 1 μM indo-1-AM ester, and then stained with FITC-conjugated anti-B220 antibody to identify B cells, before [Ca^{2+}]; responses were assessed as described in the legend to Fig. 1. All results represent at least three experiments.

Antibody (IgM) ligation was significantly less intense than with BCR ligation alone (Fig. 3A) suggested that CD19/BCR ligation may abrogate the phosphorylation of negative regulatory molecules involved in [Ca^{2+}]; responses. For example, Lyn^{−/−} B cells hypophosphorylate CD22 and multiple other cellular proteins, have augmented [Ca^{2+}]; responses, and demonstrate a hyperresonspive phenotype (36). Tyrosine-phosphorylated CD22 terminates BCR- and CD19-induced signals through the recruitment of SHIP1 and Src homology-2 domain-containing inositol polyphosphate 5 phosphatase (SHIP), intracellular phosphatases with inhibitory functions (28, 37–43). CD19 ligation alone reduced basal CD22 phosphorylation (data not shown), and CD19 ligation dramatically inhibited BCR-induced CD22 phosphorylation (Fig. 3F). Simultaneous CD19 and BCR ligation also significantly reduced the amount of phosphorylated CD22 that coprecipitated with SHP1 to 7±4% of the levels induced by IgM ligation alone (Fig. 3F; n=4). The 140 kDa phosphoprotein associated with SHP1 was verified as CD22 by preclearence of the cell lysates with an anti-CD22 antibody, which depleted this band (data not shown). In addition, SHP1 did not coprecipitate this 140-kDa protein when precipitated from CD22-deficient B cells, as published previously (44). Thus, simultaneous CD19 and BCR ligation uncoupled at least one of the major negative regulatory pathways downstream of BCR engagement.

CD19 Amplification of [Ca^{2+}]; Responses Requires CD22 and Lyn Expression—To determine whether CD22 expression influenced the exaggerated [Ca^{2+}]; responses induced by BCR and CD19 ligation, [Ca^{2+}]; responses were assessed in CD22^{−/−} B cells. Remarkably, the [Ca^{2+}]; responses observed in CD22^{−/−} B cells following BCR ligation alone were identical to the optimal [Ca^{2+}]; responses induced with simultaneous CD19 and BCR ligation in wild type B cells (Fig. 5A). BCR-induced [Ca^{2+}]; responses in CD22^{−/−} B cells were higher and lower when 40 and 10 μg/ml concentrations of anti-IgM antibody were used, respectively. However, BCR-induced [Ca^{2+}]; responses in CD22^{−/−} B cells were not amplified by simultaneous CD19 ligation using antibody at 10 μg/ml (data not shown). Because CD22 expression was required for CD19 to augment BCR-induced [Ca^{2+}]; responses, these results are consistent with simultaneous CD19 and BCR ligation uncoupling CD22 from its negative regulatory role.

Lyn is the primary tyrosine kinase that phosphorylates SHP1-binding sites on CD22 (45). Therefore, [Ca^{2+}]; responses were also assessed in Lyn-deficient (Lyn^{−/−}) B cells following BCR ligation. The augmented [Ca^{2+}]; responses observed in Lyn^{−/−} B cells following BCR ligation alone were similar to the BCR-induced [Ca^{2+}]; responses in CD22^{−/−} B cells and optimal [Ca^{2+}]; responses induced in wild type B cells by simultaneous CD19 and BCR ligation (Fig. 5A). CD19 ligation using antibody at 10 μg/ml did not further amplify BCR-induced [Ca^{2+}]; responses in Lyn^{−/−} B cells. These results suggest that simultaneous CD19 ligation may alter the quality of BCR-mediated signaling by abrogating Lyn’s ability to phosphorylate CD22. Therefore, synergistic CD19 and BCR-induced [Ca^{2+}]; responses may result from down-regulation of CD22 phosphorylation and function.

[Ca^{2+}]; Responses in B Cells that Overexpress CD19—B cells from CD19TG mice that overexpress CD19 3-fold have intrinsically augmented CD19 function and are hyperresponsive to transmembrane signals (7, 16). This allowed us to assess the effects of augmented CD19-generated signals on BCR-induced [Ca^{2+}]; responses independent of CD19 ligation by antibody. BCR-induced [Ca^{2+}]; responses were accelerated and augmented in CD19TG B cells (Fig. 6). CD19TG B cells also generated accelerated and augmented [Ca^{2+}]; responses following CD19 ligation (Fig. 6). Following BCR ligation, tyrosine phosphorylation of most cellular proteins was markedly reduced in CD19TG B cells, although some cellular proteins were phosphorylated normally or with faster kinetics (Fig. 7A). Notably, Lyn phosphorylation and kinase activity were higher in unstimulated CD19TG B cells, with faster kinetics of activation (Fig. 7B). Tyrosine phosphorylation of other Src family PTKs was not increased in CD19TG B cells relative to wild type littermates, before or after BCR ligation (data not shown). Analogous to results with BCR and CD19 co-stimulation in wild type B cells, tyrosine phosphorylation of Syk was slightly lower in CD19TG B cells than in wild type B cells after BCR ligation (data not shown).

In contrast to augmented Lyn phosphorylation in B cells overexpressing CD19, constitutive and BCR-induced CD22 phosphorylation was modest in CD19TG B cells relative to wild type B cells (Fig. 7, A and C). In addition, the amount of phosphorylated CD22 associated with SHP1 in resting and activated CD19TG B cells was markedly reduced when compared with wild type B cells (Fig. 7D). The two phosphoproteins, 140 and 130 kDa, precipitated from CD19TG B cells by SHP1 represent different CD22 phosphorylation states as revealed using a CD22 cytoplasmic domain-specific antibody as described previously (28). Anti-CD22 antibody preclearence of the cell lysates depleted these bands (data not shown). These characteristics of CD19-overexpressing B cells mimic what was observed for wild type B cells following simultaneous BCR and CD19 ligation. Although CD19TG B cells have decreased surface IgM density compared with wild type B cells (16), decreased overall protein-tyrosine phosphorylation and decreased CD22 phosphorylation were not explained by IgM density because CD19TG B cells from mice expressing high levels of an IgM transgene generated similar results (data not shown). Thus, CD22 phosphorylation was reduced in B cells overexpressing CD19, which may have contributed to their enhanced [Ca^{2+}]; responses. In addition, the results obtained with B cells that overexpressed CD19 were similar to those obtained in wild type B cells following CD19 ligation. Therefore, it is likely that these observations reflect augmented CD19 function rather than the effects of mAb treatment alone.

Simultaneous CD19/BCR Ligation Can Negatively Regulate [Ca^{2+}]; Responses—B cell [Ca^{2+}]; responses were enhanced maximally by anti-IgM F(ab’)2 antibody fragments (40 μg/ml) in combination with anti-CD19 antibody at 10 μg/ml (Fig. 2A). Unexpectedly, the initial peak of the [Ca^{2+}]; response was decreased and delayed when more anti-CD19 antibody (20 μg/ml) was added with anti-IgM antibody (40 μg/ml), but was still higher than with BCR ligation alone (Fig. 2B). However, CD19 ligation using 40 μg/ml of antibody reduced BCR-induced [Ca^{2+}]; responses to levels equivalent to CD19 ligation alone.
The ability of optimal CD19 ligation to suppress BCR-induced [Ca^{2+}]i responses did not require CD22 or Lyn expression. In CD22^-/- B cells, CD19 ligation using 40 μg/ml of antibody reduced BCR-induced [Ca^{2+}]i responses to levels equivalent to optimal CD19 ligation alone (Fig. 5B). Thus, the ability of CD19 to inhibit BCR-induced [Ca^{2+}]i responses following optimal CD19 engagement does not appear to involve enhanced CD22 or Lyn activities. Rather, simultaneous BCR and CD19 ligation with optimal concentrations of both antibodies (40 μg/ml) inhibited Lyn, CD79a, Syk, and CD22 phosphorylation to levels induced by ligation of CD19 (40 μg/ml) alone (Fig. 3 and data not shown). Therefore, optimal ligation of both the BCR and CD19 abrogated both BCR-induced [Ca^{2+}]i responses and the induced phosphorylation of CD22, Lyn, Syk, and CD79a.

DISCUSSION

The current study demonstrates that signal transduction pathways activated by independent CD19 and BCR cross-linking synergize to generate augmented [Ca^{2+}]i responses (Figs. 1 and 2A) without a requirement for CD19-BCR co-ligation (Fig. 2A). This supports a model where CD19 ligation can influence signaling thresholds of B cells independent of antigen receptor engagement or regardless of antigen receptor specificity (1, 46). This conclusion is supported by the phenotypes of CD19-deficient and -overexpressing mice (1). Although IgM and heparin sulfate binding to CD19 and CD3 binding to the CD21 component of the CD19 complex are the only currently characterized ligands for the CD19 complex (47 and 48), the ability of CD19 ligation to lower signaling thresholds without a requirement for intimate physical association with the BCR complex suggests that CD19 ligands may regulate B cell function regardless of their antigen specificity. Nonetheless, the finding that simultaneous CD19 and BCR ligation resulted in an overall decrease in B cell protein-tyrosine phosphorylation relative to phosphorylation induced by IgM cross-linking alone was unexpected (Fig. 3A). Overall, BCR-induced protein-tyrosine phosphorylation was also reduced in B cells from mice that overexpress CD19 (Fig. 7A). Consistent with this, simultaneous BCR and CD19 ligation reduces Vav tyrosine phosphorylation (44), whereas independent BCR or CD19 ligation augments Vav phosphorylation and CD19-Vav complex formation (30, 32, 44). Thus, rather than augmenting overall B cell kinase activity, simultaneous CD19 ligation inhibited specific kinase pathways downstream of BCR engagement.

The recent observations that CD19 efficiently recruits Src family PTKs (11 and 12) and that phosphorylated CD19 preferentially localizes within detergent-insoluble lipid raft microdomains of B cells may provide a mechanistic link to these findings.
CD19 Ligation Uncouples CD22 Negative Regulation

44825

Fig. 7. Signal transduction in CD19TG B cells that overexpress CD19. A, protein-tyrosine phosphorylation in B cells (5 × 10^6/lane) from wild type and CD19TG mice was assessed after BCR ligation as in Fig. 5A. BCR-induced tyrosine phosphorylation of CD22 precipitated using anti-CD22 antibody (B) or associated with SHP1 (C) was assessed as in Figs. 3, B and C. D. Lyn tyrosine phosphorylation and kinase activity following BCR ligation was assessed as in Fig. 3E. An asterisk indicates a mean significantly different from those of unstimulated B cells, *p < 0.05. In all cases, purified splenic B cells were incubated with either medium alone (time 0) or with anti-IgM F(ab')2 antibody fragments (40 μg/ml) for the indicated times. All results represent those obtained with B cells from at least six littermate pairs of mice.

Signaling domains (49 and 50) provides a mechanistic explanation for why simultaneous BCR and CD19 ligation augments [Ca^{2+}], responses (Fig. 2) yet down-regulates B cell protein-
tyrosine phosphorylation (Fig. 3). Lyn, the primary Src family PTK in B cells, preferentially localizes within lipid rafts and plays a critical role in regulating BCR, CD19, and CD22 function (45). Because CD19, CD22, and Lyn expression are genetically titrated in B cells (51), BCR signaling is significantly influenced by alterations in the expression and functional pools of these molecules (7, 8, 14, 45, 52, and 53). Lyn and other Src family PTKs become activated following BCR engagement with increased binding to phosphorylated CD19 and the BCR (11, 54–57). In turn, phosphorylated CD19 functions as an intrinsic adapter protein for Lyn binding and the amplification of Src family PTK activity (11 and 12). In agreement with this, simultaneous CD19 engagement during BCR signaling significantly augmented CD19/Lyn complex formation (Fig. 3B). CD19 is likely to regulate the spatial distribution of Lyn at the membrane/cytoplasm interface because both Lyn and tyrosine-phosphorylated CD19 preferentially localize within lipid rafts, and ligation of the CD19 complex results in a translocation of CD19 into lipid rafts regardless of concomitant BCR ligation (49 and 50). The ability of phosphorylated CD19 to efficiently recruit and form CD19/Lyn complexes may sequester functionally active Lyn to a sufficient degree that multiple downstream signaling pathways and CD22 phosphorylation are inhibited (11, 58). Although the amount of Lyn physically associated with CD19 at any instance only represents a small percentage of total cellular Lyn (11), the proportion of cellular Lyn that is functionally active or available for the CD19 or BCR signaling pathways is unknown. Therefore, small changes in the available pool of Lyn may be of profound importance. Consistent with the hypothesis that CD19 cross-linking augments [Ca^{2+}], responses by sequestering Lyn away from CD22 and other negative regulatory molecules, BCR-induced [Ca^{2+}], responses are significantly enhanced in Lyn^−/− B cells (Fig. 5B, Ref. 36). Thus, BCR, CD19, and Lyn cross-talk are likely to be precisely balanced to regulate [Ca^{2+}], responses and B cell function.

Effector molecules participating in BCR-induced [Ca^{2+}], responses include PI 3-kinase and CD22, which are recruited to or regulated by tyrosine-phosphorylated CD19 (31, 58). PI 3-kinase binding does not appear to be the sole mechanism by which CD19 augments BCR signaling because inhibiting PI 3-kinase activity did not ablate the ability of CD19 to augment BCR-induced [Ca^{2+}], responses (Fig. 4). Current studies suggest that CD19 ligation may enhance BCR-induced [Ca^{2+}], responses by sequestering Lyn and thereby inactivating selective downstream inhibitory signaling pathways, including CD22 phosphorylation (Fig. 3F). In support of this, Lyn is primarily responsible for CD22 phosphorylation (28, 45) and CD19 ligation during BCR engagement down-regulated CD22 phosphorylation and SHP1 recruitment by CD22 (Fig. 3, H and I). In addition, BCR-induced [Ca^{2+}], responses were enhanced in B cells that overexpressed CD19 (Fig. 6), whereas CD22 phosphorylation and SHP1 recruitment by CD22 following BCR ligation were inhibited (Fig. 7, C and D). Augmented BCR-induced [Ca^{2+}], responses are a hallmark of CD22-deficient B cells (Fig. 5A, Refs. 26, 59–61), but BCR-induced [Ca^{2+}], responses in CD22- or Lyn-deficient B cells were not augmented by simultaneous CD19 ligation (Fig. 5A). Although it remains unknown exactly how CD22 exerts inhibitory effects on B cell [Ca^{2+}], responses, CD22 regulation is likely to involve recruitment of the SHP1 and SHIP phosphatases (28, 36, 52, 62, and 63). Lyn is reported to be a target for SHP1 dephosphorylation (64), consistent with the observation that Lyn phosphorylation may be increased by simultaneous CD19 and BCR engagement (Fig. 3C). Thus, simultaneous CD19 and BCR engagement may augment and/or maintain Lyn activity through multiple mechanisms; CD19 amplification of Lyn ki-
nase activity, decreased CD22 phosphorylation, and diminished SHP1 and SHIP binding to CD22. CD22 may also be excluded from lipid rafts, which may enhance CD19/Lyn phosphorylation if these proteins remain within lipid rafts following CD19 engagement. Consistent with these observations, CD19 phosphorylation was equally high following BCR or simultaneous CD19 and BCR ligation in CD22−/− B cells. Thus, the ability of CD19 ligation to down-regulate phosphorylation of CD22, and perhaps other negative regulatory molecules, may enhance CD19 function.

CD19 was originally regarded to be both a positive and negative regulator of BCR signal transduction because CD19 cross-linking could either augment or inhibit BCR signaling and [Ca²⁺], responses (21–24). Consistent with this, high valency CD19 ligation inhibited BCR-induced [Ca²⁺], responses, whereas lower valency ligation augmented [Ca²⁺], responses (Fig. 2, B and C). In addition, antibodies reactive with the CD81 component of the CD19 complex are potent inhibitors of B cell proliferation (65). Thus, CD19 may serve both positive and negative roles, depending on its level of engagement. High valency CD19 ligation may efficiently sequester the available pool of functional Lyn because Syk and CD79a phosphorylation were not induced when CD19 was engaged using anti-CD19 antibody at high concentrations. Under these conditions, Lyn, Syk, and CD79a were only phosphorylated at levels equivalent to those induced by CD19 ligation (40 μg/ml) alone (Fig. 3 and data not shown). This proposed mechanism explains why high valency CD19 ligation inhibited BCR-induced [Ca²⁺], responses (Fig. 2C), even in the absence of CD22 expression (Fig. 5B). That high valency CD19 ligation inhibited BCR-induced [Ca²⁺], responses in Lyn−/− B cells (Fig. 3B) may be explained by the observation that CD19 binds and amplifies Src family PTKs in B cells (61, 57, and 58). Alternatively, CD19 cross-linking by antibody may sequester CD19 away from the BCR complex and thereby down-regulate signaling. Arguing against this possibility is the observation that tyrosine phosphorylation of cellular proteins was decreased in CD19 overexpressing B cells following BCR engagement in the absence of CD19 ligation (Fig. 7A). Moreover, CD19 ligation with high antibody levels reduced BCR-induced [Ca²⁺], responses to the levels induced by CD19 ligation alone. Because anti-IgM-induced [Ca²⁺], responses are near normal in CD19-deficient B cells (14), it is not clear how antibody sequestration of CD19 away from IgM would have a more profound effect on BCR-induced [Ca²⁺], than the total absence of CD19.

Thus, high valency CD19 ligation may sequester Lyn and other Src family PTKs to a sufficient degree that multiple downstream signaling pathways required for [Ca²⁺], responses are inhibited.

These results collectively demonstrate that CD19 engagement may alter the quality of BCR-mediated signaling without a requirement for co-ligation of these two receptor complexes. The ability of CD19 to amplify Lyn kinase activity (11 and 12) in combination with its potential to extinguish CD22 negative regulation may explain its potent synergy with BCR signal transduction. Thus, the current results suggest that the BCR, CD19, and other positive and negative regulatory cell surface proteins may turn on and off other receptor functions by recruiting or sequestering a shared pool of Lyn. Sequestration of a limiting supply of Lyn in mast cells is a proposed basis for antagonistic FceRI function (66). Undoubtedly, other receptors are likely to sequester limited supplies of downstream effector molecules after engagement and may thereby utilize a similar strategy to regulate intercellular signaling and direct the activation of downstream pathways.

Acknowledgments—We thank Drs. C. Lowell, M. Grove, and L. Matsuuchi for providing mice and reagents for these studies.
CD19 Ligation Uncouples CD22 Negative Regulation

Access the most updated version of this article at doi: 10.1074/jbc.M107559200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 66 references, 39 of which can be accessed free at http://www.jbc.org/content/276/48/44820.full.html#ref-list-1