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Regulation of MHC Class II Signal Transduction by the B Cell Coreceptors CD19 and CD22

Kevin R. Bobbitt* and Louis B. Justement†

The major histocompatibility class II heterodimer (class II)3 is expressed on the surface of both resting and activated B cells. Although it is clear that class II expression is required for Ag presentation to CD4+ T cells, substantial evidence suggests that class II serves as a signal transducing receptor that regulates B cell function. In ex vivo B cells primed by Ag receptor (BCR) cross-linking and incubation with IL-4, or B cell lines such as K46-17μm, class II ligation leads to the activation of protein tyrosine kinases, including Lyn and Syk and subsequent phospholipase Cγ-dependent mobilization of Ca2+. In this study, experiments demonstrated reciprocal desensitization of class II and BCR signaling upon cross-linking of either receptor, suggesting that the two receptors transduce signals via common processes and/or effector proteins. Because class II and BCR signal transduction pathways exhibit functional similarities, additional studies were conducted to evaluate whether class II signaling is regulated by BCR coreceptors. Upon cross-linking of class II, the BCR coreceptors CD19 and CD22 were inducibly phosphorylated on tyrosine residues. Phosphorylation of CD22 was associated with increased recruitment and binding of the protein tyrosine phosphatase SHP-1. Similarly, tyrosine phosphorylation of CD19 resulted in recruitment and binding of Vav and phosphotyrosinolysine 3-kinase.

Finally, co-cross-linking studies demonstrated that signaling via class II was either attenuated (CD22/SHP-1) or enhanced (CD19/Vav and phosphotyrosinolysine 3-kinase), depending on the coreceptor that was brought into close proximity. Collectively, these results suggest that CD19 and CD22 modulate class II signaling in a manner similar to that for the BCR. The Journal of Immunology, 2000, 165: 5588–5596.

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3 Abbreviations used in this paper: class II, MHC class II; PTP, protein tyrosine phosphatase; BCR, B cell Ag receptor; PKC, protein kinase C; PTK, protein tyrosine kinase; PLCγ, phospholipase Cγ; IP3, 1,4,5-inositol triphosphate; PI 3-K, phosphotyrosinolysine 3-kinase; ECL, enhanced chemiluminescence; GEM, glycolipid-enriched microdomain.
II signaling. CD19 is a member of a multimeric complex comprised of CD21, CD81, and Leu-13 and has been shown to enhance signaling via the BCR (35–38). Tyrosine phosphorylation of the cytoplasmic domain of CD19 generates phosphotyrosine motifs that recruit Vav and phosphatidylinositol 3-kinase (PI 3-K), as well as other effector proteins (39–41). CD22 is a B-cell restricted, 140-kDa glycosylated transmembrane protein that is inducibly phosphorylated on tyrosine in response to BCR ligation. Phosphorylation of CD22 mediates the recruitment of several effector proteins including PLCγ, PI 3-K, Syk, and the protein tyrosine phosphatase (PTP) SHP-1 (42–44). Recruitment and activation of SHP-1 by CD22 has been demonstrated to attenuate signal transduction through the BCR (45–47).

In this study, CD19 and CD22 were observed to become tyrosine phosphorylated following class II ligation, resulting in the recruitment of specific effector proteins. As a result, signaling was influenced either negatively (CD22/SHP-1) or positively (CD19/ Vav and PI 3-K) when class II was coligated with these coreceptors. Collectively, the results obtained suggest that CD19 and CD22 can modulate class II- and BCR-dependent signaling in a similar manner.

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Materials and Methods

Animals
C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All experiments were performed using mice between the ages of 6 and 8 wk.

Biological reagents
The mAbs used in these studies were: D13.137.1 (rat IgG2a, anti-mouse MHC class II, Iaαβ haplotype), Bet-2 (rat anti-mouse μ, heavy chain), B76 (rat IgG1, anti-mouse μ heavy chain), RG7/9.1 (mouse IgG2b, anti-rat κ light chain), and CY34.1 (mouse IgG1, anti-mouse CD22). The mAbs were purified using protein G-Sepharose 4B fast flow beads (Amersham Pharmacia Biotech) and were biotinylated using N-hydroxysuccinimidobiotin (Sigma, St. Louis, MO) as described previously (48). The Abs PTPIC (mouse IgG1, anti-PI 3-K; Transduction Laboratories, Lexington, KY), 1D3 (rat IgG2a, anti-mouse CD19; PharMingen, San Diego, CA), PE-conjugated CY34.1 (mouse IgG1, anti-mouse CD22; PharMingen), anti-Vav (mouse anti-human Vav; Upstate Biotechnology, Lake Placid, NY), anti-PI 3-K (mouse IgG1, anti-human PI 3-K; Transduction Laboratories), anti-IgG/A/F (Fab′2) goat anti-mouse IgG, IgA, IgM; Organon Teknika, West Chester, PA), and 4G10 coupled to HRP (mouse IgG2b, anti-phosphotyrosine; Upstate Biotechnology) were purchased for these studies. Dr. Robert Carter (Department of Medicine, University of Alabama at Birmingham, Birmingham, AL), generously provided mouse anti-CD19 cytoplasmic domain mAb. The secondary Abs used in these studies included goat anti-mouse IgG coupled to HRP and goat anti-rabbit IgG coupled to HRP (BioSource International, Camarillo, CA). Additional reagents used in these studies included NeutrAvidin (Pierce, Rockport, IL) and streptavidin conjugated with PE (BioSource International). Mouse IL-4 was purchased from R&D Systems (Minneapolis, MN).

Splenic B cells and cell lines
Resting splenic B cell were purified as previously described (23). Briefly, after preparing a single cell suspension of splenocytes, RBC were lysed after preparing a single cell suspension of splenocytes, RBC were lysed by guest on August 31, 2017 http://www.jimmunol.org/ Downloaded from http://www.jimmunol.org/ by guest on August 31, 2017

Measurement of calcium mobilization
Either splenic B cells or K46 cells (1 × 10^6/ml) were loaded with Indo-1 AM at a final concentration of 5 μM. Indo-1-loaded cells were analyzed using a Becton Dickinson FACSF luorescence flow cytometer (San Jose, CA) equipped with an Enterprise laser from Coherent (Santa Clara, CA) set for excitation at ~364 nm at a power setting of 60 mW. Fluorescence emissions were separated by a 505-nm short pass beam splitter into two component emissions by passage through 405- and 485-nm centered 10-nm band pass filters to detect violet and blue, respectively. The ratio of emissions was calculated, and a plot was constructed of fluorescence ratio vs time. Before analysis, cells were incubated with biotinylated anti-class II mAb (D3.137.5.7, 20 μg/ml) for 15 min at room temperature. The cells were washed once in room temperature PBS, resuspended in IMDM with 5% FBS (1 ml/sample), and added to equilibrate for 15 min at 37°C under 5% CO2. The cells were stimulated for various times with NeutrAvidin (20 μg/ml). Stimulation of cells with either anti-class II mAb or NeutrAvidin alone did not elicit a signaling response as determined by measurement of calcium mobilization (data not shown). The effect of signaling through class II was compared with that observed when cells were stimulated through the BCR (goat anti-mouse IgG/A/M Fab′2, 5 μg/ml), or with pervanadate. For consistency, cells stimulated through the BCR or with pervanadate were handled in the same manner as those stimulated with anti-class II mAb and NeutrAvidin. After stimulation, samples were washed twice in 7 ml of ice-cold PBS and lysed in 0.5 ml of lysis buffer (25 mM HEPES, 150 mM NaCl, pH 7.8, 10 mM EDTA, 1 mM EGTA, 0.1 mM Na3VO4, and 1% Nonidet P-40). Cells were lysed for 1 h on ice, and the lysates were centrifuged at 12,000 × g for 15 min at 4°C to remove debris. Lysates were preincubated with protein G-Sepharose beads (Vav, PI 3-K, CD19, SHP-1) or by the addition of Ab coupled directly to Sepharose 4B beads (CD22). Each immunoprecipitation step was performed for 1 h at 4°C with rotation. Immune complex-coated beads were collected and washed four times with lysis buffer containing 0.2% Nonidet P-40. The beads were resuspended in 50 μl of reducing SDS-PAGE sample buffer, boiled for 5 min, and centrifuged at 12,000 × g for 5 min.

For each condition, the proteins contained in 15 μl of SDS-PAGE sample buffer were separated on 8% acrylamide gels using SDS-PAGE and were transferred to Hybond-ECL nitrocellulose membranes. Membranes were blocked in either 3% BSA (Sigma, St. Louis, MO) with 10% nonfat dry milk in TBST (Vav, PI 3-K, CD19, SHP-1) for 1 h at room temperature. The membranes were then washed five times with TBST. To detect inducible tyrosine phosphorylation of CD22 and CD19, blots were incubated with anti-phosphotyrosine (PY1) mAb (1:000, 4G10-HPR) for 1 h at room temperature. The blots were washed in TBST and incubated with goat anti-mouse IgG (1:10,000) for 1 h at room temperature. Tyrosine phosphorylation of CD19 and CD22 was visualized using enhanced chemiluminescence (ECL) according to the manufacturer’s instructions (Super Signal chemiluminescent substrate; Pierce). To detect binding of SHP-1 to CD22, or Vav and PI 3-K to CD19, the appropriate blots were incubated by stripping them in stripping buffer (10 mM Tris, pH 2.3, 150 mM NaCl) at 70°C for 1 h, after which they were blocked with 5% powdered defatted milk (BioRad). Blots were then incubated with anti-mouse IgG (1:10,000) or anti-IgG/A/F (1:250), anti-Vav Ab (1: 250), or anti-PI 3-K Ab (1:300) for 1 h at room temperature. Each blot was washed in TBST and incubated with HRP-conjugated goat anti-mouse IgG (1:10,000) for 1 h at room temperature. The membranes were then washed and developed using ECL to visualize binding of effector proteins. In select CD19 experiments, membranes were stripped and reprobed with mouse anti-CD19 cytoplasmic tail mAb followed by the addition of HRP-conjugated goat anti-mouse IgG to confirm equal recovery and loading of CD19.

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Biological reagents
The mAbs used in these studies were: D13.137.1 (rat IgG2a, anti-mouse MHC class II, Iaαβ haplotype), Bet-2 (rat anti-mouse μ, heavy chain), B76 (rat IgG1, anti-mouse μ heavy chain), RG7/9.1 (mouse IgG2b, anti-rat κ light chain), and CY34.1 (mouse IgG1, anti-mouse CD22). The mAbs were purified using protein G-Sepharose 4B fast flow beads (Amersham Pharmacia Biotech) and were biotinylated using N-hydroxysuccinimidobiotin (Sigma, St. Louis, MO) as described previously (48). The Abs PTPIC (mouse IgG1, anti-PI 3-K; Transduction Laboratories, Lexington, KY), 1D3 (rat IgG2a, anti-mouse CD19; PharMingen, San Diego, CA), PE-conjugated CY34.1 (mouse IgG1, anti-mouse CD22; PharMingen), anti-Vav (mouse anti-human Vav; Upstate Biotechnology, Lake Placid, NY), anti-PI 3-K (mouse IgG1, anti-human PI 3-K; Transduction Laboratories), anti-IgG/A/F (Fab′2) goat anti-mouse IgG, IgA, IgM; Organon Teknika, West Chester, PA), and 4G10 coupled to HRP (mouse IgG2b, anti-phosphotyrosine; Upstate Biotechnology) were purchased for these studies. Dr. Robert Carter (Department of Medicine, University of Alabama at Birmingham, Birmingham, AL), generously provided mouse anti-CD19 cytoplasmic domain mAb. The secondary Abs used in these studies included goat anti-mouse IgG coupled to HRP and goat anti-rabbit IgG coupled to HRP (BioSource International, Camarillo, CA). Additional reagents used in these studies included NeutrAvidin (Pierce, Rockport, IL) and streptavidin conjugated with PE (BioSource International). Mouse IL-4 was purchased from R&D Systems (Minneapolis, MN).
Receptor-mediated desensitization assay

K46 cells (1 × 10^6/ml) were loaded with Indo-1 AM at a final concentration of 5 µM. Analysis of class II-mediated and/or IgM-mediated Ca^{2+} mobilization was performed as described previously (37). Before analysis, cells were incubated with biotinylated anti-class II mAb (D3.137, 20 µg/ml) for 15 min at room temperature, then washed. To measure BCR-mediated desensitization of class II signaling, anti-class II coated cells were analyzed using flow cytometry as previously described to establish the baseline level of intracellular free Ca^{2+}. Once the baseline had been measured, anti-IgM mAb (B76, 15 µg/ml) was added to the cells and the analysis resumed. After the response to BCR cross-linking had returned to baseline, NeutrAvidin (20 µg/ml) was added to the same sample. Additional samples, prepared as previously described, were analyzed to determine whether class II ligation leads to desensitization of BCR signaling.

For these experiments, cells were analyzed to determine the baseline level of free intracellular Ca^{2+} after which they were stimulated by first cross-linking the biotinylated anti-class II mAb (D3.137.1, 20 µg/ml) with the addition of NeutrAvidin (20 µg/ml). After the response had returned to baseline, the same cells were stimulated via the addition of anti-IgM (B76, 15 µg/ml) and then analyzed immediately.

Results

MHC class II and the BCR are coupled to functionally interrelated signaling pathways

The studies presented in this paper used the K46 B lymphoma cell line to examine MHC class II signaling. This cell line is representative of an activated B lymphocyte in that cross-linking of class II leads to PTK activation and calcium mobilization in the absence of priming signals, such as anti-Ig mAb and IL-4 (24, 25). Because signaling through class II has previously been shown to involve the activation of effector proteins that are common to the BCR signal transduction pathway (23–25), studies were designed to determine whether stimulation through the BCR desensitizes class II signaling. K46 cells loaded with the calcium-sensitive dye Indo-1 were preincubated with biotinylated anti-class II mAb (D3.137.1, 20 µg/ml). Next, the baseline concentration of free intracellular Ca^{2+} was established for each sample, and the cells were stimulated by the addition of anti-IgM mAb (B76, 15 µg/ml), after which the analysis was resumed immediately. The response to BCR cross-linking consistently generated a large calcium flux response (Fig. 1). Once the response had returned to baseline, the same cells were restimulated by the addition of NeutrAvidin to cross-link class II receptors bound with biotinylated anti-class II mAb. Subsequent analysis of the cells failed to detect a significant calcium mobilization response after class II ligation. When compared with the normal calcium mobilization response elicited by class II ligation alone, it was clear that signaling had been attenuated by prior cross-linking of the BCR (Fig. 1).

Additional experiments were conducted to demonstrate the reciprocal desensitization of BCR signaling in response to cross-linking of class II. K46 cells were loaded with Indo-1 and preincubated with anti-class II mAb. Measurements to establish the Ca^{2+} baseline in unstimulated cells were taken, after which the cells were stimulated by ligation class II through the addition of NeutrAvidin. The analysis was resumed immediately to monitor the calcium mobilization response. After the level of free intracellular Ca^{2+} returned to baseline, the same cells were restimulated by the addition of anti-IgM mAb to cross-link the BCR (Fig. 2). A modest calcium mobilization response was observed in repeated experiments. When compared with the calcium flux normally observed following stimulation of the K46 cells via the BCR, it was evident that desensitization of BCR-mediated signaling had occurred. The data indicate that significant “cross-talk” takes place between class II and the BCR. The specific mechanism responsible for desensitization of one receptor by the other remains unclear, but does not appear to be due to depletion of intracellular Ca^{2+} stores or inhibition of Ca^{2+} influx as determined by the addition of thapsigargin to cells that had previously been stimulated through either the BCR or class II (Fig. 3).

CD19 and CD22 are inducibly phosphorylated on cytoplasmic tyrosine residues following class II cross-linking

Based on the finding that class II and the BCR exhibit the ability to reciprocally desensitize one another, and therefore presumably use similar signaling pathways, it was of interest to determine whether class II signaling is regulated by coreceptors in a manner similar to the BCR. Because CD19 and CD22 have been shown to regulate signals transduced through the BCR in either a positive or negative manner, respectively (33, 34, 49), experiments were conducted to determine whether these coreceptors modulate signals transduced by class II. K46 cells were stimulated by incubation with biotinylated mAb against class II followed by the addition of NeutrAvidin. Alternatively, cells were stimulated by the addition of anti-mouse IgG/A/M polyclonal Ab to cross-link the BCR. To detect activation-dependent phosphorylation of CD22, experiments were performed in which CD22 was immunoprecipitated from cell lysates using Sepharose 4B beads conjugated to anti-CD22 mAb (NIMR-6). The resultant immune-complexes were resolved by SDS-PAGE and were analyzed by Western blotting with an anti-phosphotyrosine mAb. Tyrosine phosphorylation of CD22 was observed within 1 min in K46 cells stimulated by BCR ligation (Fig. 4). Class II cross-linking resulted in increased protein tyrosine phosphorylation of CD22 as well, and the response exhibited kinetics that were similar to those for BCR-dependent
phosphorylation. In parallel experiments, cell lysates were incubated with soluble anti-CD19 mAb, and the resultant immune-complexes were recovered by incubation with protein G-conjugated Sepharose beads. The proteins were resolved by SDS-PAGE and analyzed by anti-phosphotyrosine Western blotting. As expected, CD19 isolated from K46 cells that were stimulated through the BCR exhibited inducible tyrosine phosphorylation (Fig. 5). As was observed in the case of CD22, class II cross-linking resulted in phosphorylation of CD19 on tyrosine residues within 1 min. These results clearly demonstrate that BCR- and class II-dependent signaling leads to comparable inducible tyrosine phosphorylation of the coreceptors CD19 and CD22.

Class II-mediated tyrosine phosphorylation of CD19 and CD22 promotes recruitment of specific effector proteins

Previous studies have demonstrated that BCR-dependent tyrosine phosphorylation of CD19 and CD22 plays a direct role in their function as coreceptors through the generation of phosphotyrosine motifs in their respective cytoplasmic domains that recruit SH2 domain-containing effector proteins (39–44). Thus, it was of interest to determine whether class II-mediated phosphorylation of CD19 and CD22 promotes the recruitment of effector proteins that might be involved in modulation of class II signaling. Toward this end, K46 cells were stimulated either by cross-linking the BCR with polyclonal anti-mouse Ig or with biotinylated anti-class II mAb and NeutrAvidin for various periods of time. Subsequently, CD22 was immunoprecipitated from cell lysates by incubation with anti-CD22 mAb conjugated to Sepharose 4B beads. The immune complexes were resolved by SDS-PAGE, and one set of aliquots was analyzed by anti-phosphotyrosine Western blotting to confirm that CD19 was inducibly phosphorylated on tyrosine (Fig. 7, lower panel). In parallel samples, tyrosine phosphorylation of CD22 isolated from cells stimulated through class II was associated with inducible recruitment of SHP-1 as well. Increased association of SHP-1 with CD22 was detectable within 1 min in both BCR and class II-stimulated cells; however, the interaction appeared to be more transient in response to class II cross-linking. Thus, the functionally relevant effector protein SHP-1 is indeed recruited to CD22 in response to both BCR- and class II-mediated phosphorylation.

The basic experiment described above was repeated and the cell lysates were split into three equal aliquots. Immunoprecipitation of CD19 was then performed by incubating each sample with soluble anti-CD19 mAb and the resultant immune complexes were recovered by incubation with Sepharose beads conjugated to protein G. The immune-complex proteins for each set of aliquots were separated by SDS-PAGE, and one set of aliquots was probed with either anti-Vav or anti-PI 3-K mAb. Following incubation of the blots with the appropriate primary Ab they were incubated with goat anti-mouse Ig secondary Ab conjugated to HRP. Tyrosine phosphorylation of CD22 in response to BCR cross-linking resulted in the recruitment of the PTP SHP-1 (Fig. 6, lower panel). In parallel samples, tyrosine phosphorylation of CD22 isolated from cells stimulated through class II was associated with inducible recruitment of SHP-1 as well. Increased association of SHP-1 with CD22 was detectable within 1 min in both BCR and class II-stimulated cells; however, the interaction appeared to be more transient in response to class II cross-linking. Thus, the functionally relevant effector protein SHP-1 is indeed recruited to CD22 in response to both BCR- and class II-mediated phosphorylation.

Western blotting with anti-phosphotyrosine mAb to confirm inducible tyrosine phosphorylation of CD22 (Fig. 6, upper panel). The blot was stripped and reprobed with anti-SHP-1 mAb before the addition of goat anti-mouse Ig secondary Ab conjugated with HRP. Tyrosine phosphorylation of CD22 in response to BCR cross-linking resulted in the recruitment of the PTP SHP-1 (Fig. 6, lower panel). In parallel samples, tyrosine phosphorylation of CD22 isolated from cells stimulated through class II was associated with inducible recruitment of SHP-1 as well. Increased association of SHP-1 with CD22 was detectable within 1 min in both BCR and class II-stimulated cells; however, the interaction appeared to be more transient in response to class II cross-linking. Thus, the functionally relevant effector protein SHP-1 is indeed recruited to CD22 in response to both BCR- and class II-mediated phosphorylation.

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be seen in Fig. 7, tyrosine phosphorylation of CD19 in response to class II ligation resulted in the recruitment of effecter proteins known to have a positive influence on BCR signaling.

**Signal transduction via class II is modulated by coligation with either CD22 or CD19**

Data obtained from the previous experiments demonstrated that tyrosine phosphorylation of CD19 and CD22 following class II ligation results in the recruitment of specific effector proteins that are known to modulate signal transduction via the BCR. However, it remained to be determined whether CD19 and CD22 could indeed affect class II signaling through the recruitment of these effector proteins. Thus, experiments were designed to investigate this possibility in which the effect of coligating either CD19 or CD22 with class II was assessed by monitoring calcium mobilization. K46 cells loaded with Indo-1 were incubated with a sub-optimal concentration of biotinylated anti-class II mAb in the presence or absence of varying concentrations of biotinylated anti-CD22 mAb. Unbound Ab was removed by washing and the basal concentration of intracellular free Ca\(^{2+}\) was measured. Next, the cells were stimulated by the addition of NeutrAvidin to cross-link class II alone or to effect co-cross-linking of CD22 and class II. Coligation of CD22 with class II resulted in a reproducible decrease in calcium mobilization that was dependent on the concentration of anti-CD22 mAb used when compared with the response generated by class II ligation alone (Fig. 8). Thus, SHP-1 recruitment by CD22 appears to exert a negative effect on class II signaling in a manner similar to that observed for BCR signaling. The effect that coligation of CD19 has on class II signaling was assessed by repeating the experiment described above using Indo-1-loaded K46 cells and substituting biotinylated anti-CD19 mAb for the biotinylated anti-CD22 mAb. Coligation of CD19 with class II, via the addition of NeutrAvidin, resulted in a significant increase in calcium mobilization when compared with class II ligation alone (Fig. 9). Because CD19 ligation alone can lead to

**FIGURE 4.** Cross-linking of class II results in tyrosine phosphorylation of CD22. K46 cells (2.5 × 10\(^7\)/sample) were stimulated either by cross-linking the BCR or class II. Cells were incubated with biotinylated anti-class II mAb (Anti-Ia, D3.137.5.7, 20 μg/ml) or medium alone, after which they were washed. To induce signaling through class II, cells incubated with anti-class II mAb were stimulated by the addition of NeutrAvidin (20 μg/ml). To induce signaling through the BCR, cells incubated in medium alone were stimulated by the addition of F(ab')\(_2\) of goat anti-mouse IgG/A/M (5 μg/ml). As a positive control, cells were incubated with pervanadate to induce tyrosine phosphorylation of CD22 in a receptor-independent manner. CD22 was immunoprecipitated from precleared lysates by the addition of Sepharose 4B beads conjugated with anti-CD22 mAb (CY34.1). After boiling, the beads in SDS-PAGE sample reducing buffer, the proteins contained in 15 μl of supernatant were separated on 8% acrylamide gels using SDS-PAGE and transferred to Hybond-ECL nitrocellulose membranes. The membranes were blocked in 3% blot qualified BSA and subsequently incubated with HRP-conjugated anti-phosphotyrosine mAb (4G10, 1:3000). The proteins were visualized using ECL.

**FIGURE 5.** Cross-linking of class II results in tyrosine phosphorylation of CD19. K46 cells (2.5 × 10\(^7\)/sample) in IMDM supplemented with 5% FBS were stimulated either through the BCR or class II as previously described. CD22 was immunoprecipitated from precleared lysates by the addition of Sepharose 4B beads conjugated with anti-CD22 mAb (CY34.1). The immune-complex proteins from each sample were separated on 8% acrylamide gels using SDS-PAGE and transferred to Hybond-ECL nitrocellulose membranes. The membranes were blocked in 3% blot qualified BSA, washed five times in TBST, and incubated with HRP-conjugated anti-phosphotyrosine mAb (4G10, 1:3000). The proteins were visualized using ECL.

**FIGURE 6.** Tyrosine phosphorylation of CD22 in response to class II signaling induces recruitment of SHP-1. K46 cells (2.5 × 10\(^7\)/sample) in IMDM supplemented with 5% FBS were stimulated either through the BCR or class II as previously described. CD22 was immunoprecipitated from precleared lysates by the addition of Sepharose 4B beads conjugated with anti-CD22 mAb (CY34.1). The immune-complex proteins from each sample were separated on 8% acrylamide gels using SDS-PAGE and transferred to Hybond-ECL nitrocellulose membranes. The membranes were blocked in 3% blot qualified BSA, washed five times in TBST, and incubated with HRP-conjugated anti-phosphotyrosine mAb (4G10, 1:3000). The proteins were visualized using ECL.

**IP: Anti-CD22**

**Probe: Anti-PTyr**

**MOI WT.x 10^-3**

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**Probe: Anti-SHP-1**

**MOI WT.x 10^-3**

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**Upper panel, Analysis of CD22 tyrosine phosphorylation in K46 cells stimulated through the BCR or class II.** **Lower panel, Inducible association of SHP-1 with CD22 in response to BCR- or class II-mediated tyrosine phosphorylation of CD22.**
calcium mobilization it was important to determine whether the increase was simply an additive effect between class II- and CD19-dependent signals. Therefore, K46 cells, which had been incubated with biotinylated anti-CD19 mAb alone, were analyzed to determine the calcium mobilization response upon CD19 cross-linking by NeutrAvidin. The results demonstrated that stimulation of K46 cells through CD19 alone does not result in appreciable calcium mobilization (Fig. 9). Thus, recruitment of Vav and PI 3-kinase by CD19 appears to positively influence class II signaling in a manner similar to that for the BCR.

Parallel experiments were performed in which resting splenic B cells were incubated in the presence of anti-IgM mAb and IL-4 as previously described (23) to prime the cells. Subsequently, the primed B cells were loaded with Indo-1 AM at a final concentration of 5 μM. Before analysis, cells were incubated with varying amounts of biotinylated anti-CD22 mAb (CY34.1, 0.5–10 μg/ml) and a suboptimal amount of biotinylated anti-class II mAb (D3.137, 5 μg/ml). Additional samples were incubated in the presence of biotinylated anti-class II mAb alone. The basal concentration of intracellular free Ca²⁺ was measured for all samples just before stimulation, after which NeutrAvidin (15 μg/ml) was added either to ligate class II alone, or to coligate class II with CD22.

**Discussion**

Previous studies have documented similarities in the signal transduction pathways associated with the BCR and MHC class II. In the current study, experiments were conducted demonstrating that the BCR and class II are coupled to signaling pathways that are functionally related based on the ability of these receptors to reciprocally desensitize one another. Additionally, signaling via class II was shown to induce phosphorylation of both CD19 and CD22 resulting in the recruitment of SH2 domain-containing effector proteins known to play a role in the function of these coreceptors. Class II-mediated signaling promoted the recruitment of SHP-1 to CD22 in a phosphorylation-dependent manner, and coligation of these receptors resulted in attenuation of class II signaling. Alternatively, phosphorylation of CD19 in response to class II cross-linking promoted the recruitment of Vav and PI 3-kinase, which potentiated class II signaling in response to coligation with CD19. These studies further support the hypothesis that the BCR and class II transduce signals via a common molecular mechanism based on the ability of CD19 and CD22 to regulate signal transduction via both receptors in a similar manner.

Because class II is constitutively coupled to the PTK-dependent signaling pathway in the K46 cell line, these cells constitute a suitable model in which it is possible to test whether BCR- or class II-mediated signaling results in heterologous desensitization of the other receptor. That the BCR and class II do indeed exhibit the ability to mediate reciprocal desensitization of one another, suggests that these receptors are coupled to signaling pathways that
are functionally linked and therefore share common processes and/or elements. Reciprocal desensitization was observed within 10–15 min after stimulation of K46 cells through either the BCR or class II suggesting that desensitization results from alterations in receptor-proximal events. This is supported by the observation that desensitization of both the BCR and class II is associated with an inability to detect inducible protein tyrosine phosphorylation upon receptor cross-linking (data not shown).

Previous studies have shown that BCR desensitization is correlated with uncoupling of the Ag recognition component (membrane Ig) from the signal transducing element comprised of Ig-α and Ig-β (50). Thus, desensitization of the BCR appears to be mediated by physical uncoupling of the receptor from intracellular membrane-proximal effector proteins (i.e., PTKs). Structure/function studies have demonstrated that the ability of class II to activate PTKs and to mediate calcium mobilization is dependent on conformational changes in the transmembrane domains of both the class II PTKs and to mediate calcium mobilization is dependent on conformational studies have demonstrated that the ability of class II to activate membrane-proximal effector proteins (i.e., PTKs). Structure/functional nature of the relationship between class II and these coreceptors has yet to be elucidated from a physiological perspective. Based on the available evidence relating to class II signaling in murine B cells, CD19 and CD22 would presumably be involved in modulating class II signaling in primed B cells in which class II is functionally coupled to the activation of PTKs, phosphoinositide hydrolysis, and Ca²⁺ mobilization.

Because class II signaling appears to be mediated by a mechanism that shares numerous similarities with the BCR, it was logical to hypothesize that class II signaling may be modulated by coreceptors, as well. BCR signaling is regulated by coreceptors including CD19 and CD22, which exert opposite effects on the magnitude and duration of the signal, thereby effectively decreasing or increasing the threshold of stimulus required to drive a response, respectively. Although CD22 and CD19 play an important role in regulating BCR signaling, studies have not previously examined the possibility that either is involved in regulating signal transduction via other surface receptors expressed by the B cell. The results presented herein demonstrate that CD19 and CD22 differentially modulate class II-mediated calcium mobilization. However, the physical-functional nature of the relationship between class II and these coreceptors has yet to be elucidated from a physiological perspective. Based on the available evidence relating to class II signaling in murine B cells, CD19 and CD22 would presumably be involved in modulation of class II signaling in primed B cells in which class II is functionally coupled to the activation of PTKs, phosphoinositide hydrolysis, and Ca²⁺ mobilization.

CD22 is a sialoadhesin that recognizes sialic acid in the natural structure Siaα-2→6Galβ1→4GlcNAc (α2, 6-linked sialic acid) (51, 52). Because class II is a sialylated glycoprotein it may function as a ligand for the extracellular sialoadhesin domain of CD22 (53, 54). As class II is constitutively sialylated, CD22 might be important for attenuation of class II signals by virtue of its ability to...
constitutively interact with class II. This would presumably pre- prevent aberrant activation/differentiation of primed B cells until such time as they encounter appropriate T cell-derived signals. B cells that have processed and presented Ag in the context of class II could transduce a productive signal in response to class II cross-linking by an Ag-specific CD4+ T cell if the interaction between class II and CD22 is effectively disrupted. In this regard, it is possible that class II may be sequestered away from CD22 via one or more mechanisms. For example, class II has been shown to associate with glycolipid-enriched microdomains (GEM) in response to ligation (32) and this might physically release it from CD22-mediated interaction. It is equally possible that contact with CD4+ T cells could promote the formation of intercellular bonds mediated by binding of CD22 to sialylated proteins on the T cell. For example, previous studies have demonstrated that CD22 binds to CD45 on T cells (55, 56). The formation of an intercellular bond between CD22 and CD45 or some other sialoglycoprotein could conceivably displace class II from CD22 on the B cell. Alternatively, studies have demonstrated that the expression of α2,6-sialyltransferase in B cells is cell cycle dependent and that a B cell-tively, studies have demonstrated that the expression of conceivably displace class II from CD22 on the B cell. Alternatively, studies have demonstrated that the expression of α2,6-sialyltransferase in B cells is cell cycle dependent and that a B cell-tively, studies have demonstrated that the expression of conceivably displace class II from CD22 on the B cell. Alternatively, studies have demonstrated that the expression of α2,6-sialyltransferase in B cells is cell cycle dependent and that a B cell-tively, studies have demonstrated that the expression of conceivably displace class II from CD22 on the B cell. Alternatively, studies have demonstrated that the expression of α2,6-sialyltransferase in B cells is cell cycle dependent and that a B cell-tively, studies have demonstrated that the expression of conceivably displace class II from CD22 on the B cell. Alternatively, studies have demonstrated that the expression of α2,6-sialyltransferase in B cells is cell cycle dependent and that a B cell-tively, studies have demonstrated that the expression of conceivably displace class II from CD22 on the B cell. Alternatively, studies have demonstrated that the expression of α2,6-sialyltransferase in B cells is cell cycle dependent and that a B cell-tively, studies have demonstrated that the expression of conceivably displace class II from CD22 on the B cell. Alternatively, studies have demonstrated that the expression of α2,6-sialyltransferase in B cells is cell cycle dependent and that a B cell-tively, studies have demonstrated that the expression of conceivably displace class II from CD22 on the B cell.


