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CD45 Regulates Tyrosine Phosphorylation of CD22 and Its Association with the Protein Tyrosine Phosphatase SHP-1

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Cross-linking of CD45 induced capping and physical sequestration from CD22 leading to an increase in tyrosine phosphorylation of CD22 and SHP-1 recruitment. Additionally, CD22 isolated from a CD45-deficient B cell line exhibited increased basal/inducible tyrosine phosphorylation and enhanced recruitment of SHP-1 compared with CD22 isolated from CD45-positive parental cells. Subsequent experiments were performed to determine whether enhanced SHP-1 recruitment to CD22 is responsible for attenuation of receptor-mediated Ca$^{2+}$ responses in CD45-deficient cells. Catalytically inactive SHP-1 expressed in CD45-deficient cells interacted with CD22 and decreased phosphatase activity in CD22 immunoprecipitates to levels that were comparable to those in CD45-positive cells. Expression of catalytically inactive SHP-1 restored intracellular mobilization of Ca$^{2+}$ in response to MHC class II cross-linking, but did not affect B cell Ag receptor- or class II-mediated Ca$^{2+}$ influx from the extracellular space. These results indicate that CD45 regulates tyrosine phosphorylation of CD22 and binding of SHP-1. The data further indicate that enhanced recruitment and activation of SHP-1 in CD45-deficient cells affect intracellular mobilization of Ca$^{2+}$, but are not responsible for abrogation of receptor-mediated Ca$^{2+}$ influx from the extracellular space.

The transmembrane protein tyrosine phosphatase (PTP) CD45 plays a critical role in regulating lymphocyte development and activation (1). Studies have demonstrated that CD45 regulates the B cell Ag receptor (BCR) signaling threshold, thereby controlling both positive and negative selection (2–5). Analysis of CD45-deficient cell lines and cells derived from gene-targeted mice has demonstrated that CD45 is involved in regulating reversible tyrosine phosphorylation and thus the intrinsic sensitivity of the BCR to a given antigenic challenge (4–9). Additionally, CD45 has been shown to regulate signaling via MHC class II Ags expressed on the B cell in a manner analogous to that observed for the BCR (6). Thus, CD45 can regulate B cell development, activation, and differentiation by virtue of its ability to alter signaling thresholds associated with these receptors.

CD45 expression is required for optimal BCR- and class II-mediated activation of Src family protein tyrosine kinases (PTK), but not the PTK Syk (6, 8, 10). Indeed, BCR-mediated activation of Syk occurs in the absence of CD45, resulting in the phosphorylation and activation of phospholipase Cγ (PLCγ), which hydrolyzes phosphatidylinositol 4,5-biphosphate to produce the second messengers diacylglycerol and inositol 1,4,5-trisphosphate (IP$_3$) (6, 7). Despite the fact that IP$_3$ appears to be produced in cells that lack CD45, the downstream Ca$^{2+}$ mobilization response exhibits significant changes in both its amplitude and duration. Based on studies using the K46–17 μm A B cell lymphoma and derivative CD45-deficient mutants, receptor-proximal events associated with signal transduction through the BCR and class II are similarly affected by the loss of CD45 expression (6), although there are differences in the Ca$^{2+}$ mobilization responses elicited by stimulating B cells through these receptors. Whereas signaling through the BCR elicits a relatively normal intracellular Ca$^{2+}$ mobilization response in the absence of CD45, cross-linking of class II does not. In contrast, stimulation through either receptor fails to induce significant influx of Ca$^{2+}$ from the extracellular space (6). Besides alterations in the Ca$^{2+}$ response, Ras activation leading to signaling via the extracellular signal-related kinase 2 (ERK2) pathway is abrogated in cells that are CD45 deficient (3, 11). Clearly, then, CD45 plays an important role in regulating reversible tyrosine phosphorylation, which controls the production of second messengers (e.g., Ca$^{2+}$) and the activation of distal signaling pathways (e.g., ERK2-dependent signaling responses) that regulate gene transcription (2, 12).

The specific molecular defect responsible for attenuation of the Ca$^{2+}$ mobilization response in CD45-deficient B cells has not been clearly defined. Because CD45 is important for activation of Src family PTKs (6, 8, 10), it is possible that suboptimal Src PTK activation affects signal propagation, leading to decreased Ca$^{2+}$ responses. However, several events that are distal to Src PTK activation, such as Syk activation, PLCγ phosphorylation, and IP$_3$ production, do not appear to be significantly affected in CD45-deficient cells (6, 7). It is formally possible that Src family PTK activation is important for the activation of other effector proteins that regulate Ca$^{2+}$ mobilization. Alternatively, it is possible that the loss of CD45 leads to alterations in reversible tyrosine phosphorylation of downstream signaling proteins or coreceptors that, in turn, promote the recruitment/activation of inhibitory effector molecules that effectively attenuate the Ca$^{2+}$ response. In support
of this possibility, recent studies demonstrated that phosphorylation of the coreceptor CD22 is increased in cells that lack CD45 (6).

CD22 is a B cell–restricted transmembrane glycoprotein that functions as a coreceptor that regulates BCR-dependent signal transduction (13–16). CD22 coreceptor function is regulated by Lyn–dependent tyrosine phosphorylation of its cytoplasmic domain, creating phosphotyrosine motifs that recruit multiple SH2 domain–containing effector proteins (17–20). Recent studies suggest that CD22 can attenuate signal transduction via the BCR through its interaction with the PTP catalytic domain of SHP-1 (21, 22). In particular, studies have demonstrated that CD22, presumably through its interaction with SHP-1, can attenuate Ca\(^{2+}\) mobilization in response to BCR cross-linking (21, 22). Thus, it was of interest to further explore the relationship between CD45 and CD22 and to determine whether aberrant tyrosine phosphorylation of CD22 in CD45-deficient cells is responsible for attenuation of Ca\(^{2+}\) mobilization following cross-linking of the BCR or MHC class II. In these studies a catalytically inactive mutant of SHP-1 was expressed in CD45-deficient B cells in an attempt to determine whether competitive inhibition of endogenous SHP-1 binding to CD22 restores normal signal transduction.

**Materials and Methods**

**Biological reagents**

The mAbs used in these studies were: B76 (rat IgG, anti-mouse μ heavy chain), IA12.5 (rat IgG2b, anti-mouse IgD), D3.137.5.7 (rat IgG2a, anti-mouse MHC class II, Ia\(^{ab}\) haplotype), E3/2.3 (rat IgG2b, anti-mouse CD45), RG7/9.1 (mouse IgG2a, anti-rat κ light chain), CY34 (mouse IgGl, anti-mouse CD22.2), and NIRM-6 (rat IgGl, anti-mouse CD22). mAbs were purified using protein G-Sepharose 4B Fast Flow (Amersham-Phar-

**Cell lines**

The B lymphoma cell line K46–17 μm (K46) was provided by Dr. Michael Reth (Max-Planck Institut fur Immunobiologie, Freiburg, Germany). K46 cells were cultured in IMDM supplemented with 5% FBS (HyClone, Logan, UT), 2 mM L-glutamine, 50 μM 2-ME, 100 μg/ml streptomycin, 150 μg/ml gentamicin (Sigma) at 37°C under 5% CO\(_2\). CD45-negative variants of the K46 line were isolated after chemical mutagenesis with methanesulfonic acid ethyl ester (EMS) (Sigma) as described previously (6). Complement-mediated negative selection was used to remove CD45-positive cells, and FACS was used to enrich the resultant CD45-negative cell population. CD45-negative cells isolated by FACS were then cloned by limiting dilution, and several CD45-negative clones were examined. Two of these clones, 3S5 and 35S5, have been characterized as described previously (6), and the 35S5 cell line was used for these studies. The 35S5 cell line was transfected with either the empty pSFFV retroviral vector (vector control) or the pSFFV vector containing the cDNA encoding catalytically inactive SHP-1 (C453S mutant, SHP-1-C453S, provided by Dr. Matt Thomas, Department of Pathology, Washington University, St. Louis, MO). The SHP-1-C453S protein contains a Myc epitope tag on the carboxyterminal end to facilitate discrimination between endogenous and catalytically inactive forms of SHP-1. For transfection 100 K46 and 35S5 cells were resuspended in 500 μl of IMDM and transfected with 10 μg of pDNA using a Becton Dickinson electroporator (Mountain View, CA) with settings of 960 mF and 0.25 kV. After 48 h cells were selected in medium containing 1 mg/ml G418 (Life Technologies, Grand Island, NY). Lysates from drug-resistant transfectants were resolved by SDS–PAGE, and Western blotting was performed to detect expression of the c-Myc-tagged SHP-1-C453S mutant by sequentially probing membranes with anti-Myc Ab (9E10) followed by anti-SHP-1 Ab. Enhanced chemiluminescence (ECL) was used to visualize expression of endogenous and catalytically inactive SHP-1.

**Immunoreceptor coupling studies**

To determine whether CD22 colocalizes in the plasma membrane with CD45, K46 cells (1 × 10\(^6\)) were incubated in the presence of anti-CD45 mAb (biotinylated I3/2.3, 22 μg/ml) for 20 min on ice. The cells were washed in PBS and were incubated in PBS with Cd\(^{2+}\) and Mg\(^{2+}\)-free PBS containing 2% sodium azide and 2% FBS and were incubated with anti-CD22 mAb (PE-conjugated NIRM-6, 10 μg/ml) for 20 min on ice. Control samples were pre pared in which CD45 was not catted by performing all the incubation steps described above at 4°C. Subsequently, the cells were washed, and by cytospinning the cells onto glass slides. The cells were fixed by incubating the slides in ice-cold ethanol overnight. Fixed slides were washed and mounted in Fluoromount G (Southern Biotechnology Associates, Birmingham, AL) and viewed with a Leica/Leitz DMIRB fluorescence microscope equipped with the appropriate filter cubes for two-color immunofluorescence (Chromatechnology, Brattleboro, VT).

**Immunoprecipitation and immunoblotting**

To analyze the effect that aggregation of CD45 has on CD22 phosphorylation, K46 cells (1 × 10\(^6\)) were incubated in medium alone or in the presence of anti-CD45 mAb (biotinylated I3/2.3, 10 μg/ml) for 15 min at room temperature. Cells were then washed twice in PBS and were resuspended in 1 ml of IMDM containing 5% FBS. The cells were rest at 37°C for 20 min, and streptavidin (10 μg/ml) was added, after which cells were rest at 37°C for 1, 10, or 30 min. Control samples (NT) were incubated neither anti-CD45 nor streptavidin but were incubated at 37°C for 30 min. Reactions were stopped by the addition of ice-cold PBS containing 1 mM sodium orthovanadate.

**MHC class II- and BCR-mediated signaling events** were analyzed as described previously (6). Briefly, to analyze MHC class II-mediated signal ing, K46 cells, the CD45-negative variant 35S5, or the SHP-1-C453S transfectant of 35S5 (2.5 × 10\(^6\) cells/sample) were incubated in medium alone (NT) or in the presence of biotinylated anti-class II mAb (D3.137.5.7, 10 μg/ml) for 15 min at room temperature. Cells were then washed in PBS, resuspended in IMDM, and rested at 37°C for 20 min, followed by incubation for 1, 10, or 30 min with streptavidin (10 μg/ml). Cells (2.5 × 10\(^6\)) were harvested, rinsed, and were rest at 37°C for 20 min, and incubated with medium alone (NT) or in the presence of mAb directed against the BCR (anti-IgM, B76, 10 μg/ml) for 1, 10, or 30 min.

After incubation, cells were washed twice in 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 Na\(_2\)VO\(_4\), and 1% Nonidet P-40). Cells were incubated for 1 h on ice, and the lysates were centrifuged at 12,000 × g for 15 min at 4°C. Detergent-soluble lysates were preclarified by incubation with protein G-Sepharose (for SHP-1, c-Myc, and Lyn experiments) or RG7/9.1 bound to Sepharose 4B (for CD22 experiments) for 1 h at 4°C. Proteins to be analyzed were immunoprecipitated from preclarified lysates either by the addition of soluble Abs (SHP-1, c-Myc, and Lyn) plus protein G-Sepharose or by the addition of mAb coupled directly to Sepharose 4B beads (CD22). Samples were incubated 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 25 μl of SDS–PAGE sample reducing buffer, boiled for 4 min, and centrifuged. The proteins in 15 μl of supernatant from each sample were separated on 8% acrylamide gels by SDS–PAGE and were transferred to Hybond-ECL nitrocellulose membranes (Amersham-Pharmacia Biotech). The membranes were blocked with 10% nonfat dry milk in TBST for 1 h at room temperature and were washed five times with TBST. Next, the membranes were incubated with the appropriate primary Ab, washed five times with TBST, incubated with the appropriate HRPO-conjugated secondary Ab for 1 h at room temperature, washed five times with TBST, and visualized using ECL. In selected experiments, blots were stripped by incubation in stripping buffer (10 mM Tris (pH 2.3) and 150 mM NaCl) at 60°C for 1 h and then were washed extensively as described above. The
the addition of anti-m

established. Once a baseline measurement for intracellular free Ca$^{2+}$ was initiated. The BCR-mediated Ca$^{2+}$ influx was performed demonstrating that redistribution of CD22 within the plasma membrane of B cells can lead to alterations in the phosphorylation of other transmembrane proteins such as Igα and Igβ (31). Therefore, experiments were performed to determine whether Ab-mediated cross-linking and redistribution of CD45 affect CD22 phosphorylation and recruitment of intracellular effector proteins. First, experiments were performed to determine whether CD22 and CD45 colocalize in the membrane when CD45 is cross-linked and capped. In unstimulated B cells, both FITC-labeled CD45 and PE-labeled CD22 exhibit a uniform distribution throughout the plasma membrane (Fig. 1A). By comparison, when cells were incubated with biotinylated anti-CD45 followed by FITC-streptavidin, there was a marked capping of CD45, with little or no concurrent redistribution of CD22 in the membrane (Fig. 1B).

Next, experiments were performed to determine whether redistribution of CD45 in the plasma membrane affects CD22 phosphorylation. CD45 was cross-linked with anti-CD45 mAb (PE-conjugated NIMR-6) for 20 min on ice. Slides were prepared by cyto-spinning the cells onto glass slides, after which they were incubated in ice-cold ethanol overnight. Fixed slides were washed and mounted in Fluoromount G and viewed with a Leica/Leitz DMRB fluorescence microscope equipped with appropriate filter cubes.

membranes were then blocked with 3% blot qualified BSA (Promega, Madison, WI) in PBS, washed extensively, and incubated with anti-PTyr mAb (4G10) for 1 h. The membranes were washed and developed as described above using ECL.

Measurement of calcium mobilization

Experiments to measure CD22-associated protein tyrosine phosphatase activity were performed using Tyrosine Phosphatase Assay Kit I from Upstate Biotechnology following the manufacturer’s protocol. The samples were prepared for assay by stimulating K46 cells, 35S5 CD45-negative variants, or 35S5:SHP-1:C453S transfectants (2 × 10^7/sample) with mAbs directed against the BCR or MHC class II, and the resulting Ca$^{2+}$ mobilization response was allowed to return to baseline. Once the baseline was resolved, CaCl$_2$ was added to a final concentration of 4 mM, and measurement of extracellular Ca$^{2+}$ influx was initiated.

Measurement of CD22-associated protein tyrosine phosphatase activity

Experiments to measure CD22-associated protein tyrosine phosphatase activity were performed using Tyrosine Phosphatase Assay Kit I from Upstate Biotechnology following the manufacturer’s protocol. The samples were prepared for assay by stimulating K46 cells, 35S5 CD45-negative variants, or 35S5:SHP-1:C453S transfectants (2 × 10^7/sample) with mAbs directed against the BCR or MHC class II. The baseline measurement for intracellular free Ca$^{2+}$ was recorded demonstrating that redistribution of CD22 within the plasma membrane of B cells can lead to alterations in the phosphorylation of other transmembrane proteins such as IgG and Igβ (31). Therefore, experiments were performed to determine whether Ab-mediated cross-linking and redistribution of CD45 affect CD22 phosphorylation and recruitment of intracellular effector proteins. First, experiments were performed to determine whether CD22 and CD45 colocalize in the membrane when CD45 is cross-linked and capped. In unstimulated B cells, both FITC-labeled CD45 and PE-labeled CD22 exhibit a uniform distribution throughout the plasma membrane (Fig. 1A). By comparison, when cells were incubated with biotinylated anti-CD45 followed by FITC-streptavidin, there was a marked capping of CD45, with little or no concurrent redistribution of CD22 in the membrane (Fig. 1B).

Next, experiments were performed to determine whether redistribution of CD45 in the plasma membrane affects CD22 phosphorylation. CD45 was cross-linked with anti-CD45 mAb (biotinylated E3/2.3) plus streptavidin, and CD22 was immunoprecipitated from cell lysates. Western blotting was performed to measure tyrosine phosphorylation of CD22, revealing an increase in its tyrosine phosphorylation in conjunction with cross-linking of CD45 (Fig. 2). Because CD22 has been shown to play a role in negatively regulating BCR signal transduction through its tyrosine phosphorylation-dependent interaction with the PTP SHP-1 (22), the blot

mM HEPES (pH 7.6), 150 mM NaCl, and 0.1% Nonidet P-40), and twice in assay buffer (40 mM 2-(N-morpholino)ethanesulfonic acid and 1.6 mM DTT). The assays were performed as indicated in the protocol. An incubation time of 30 min at 30°C with phosphopeptide was determined to be optimal for measurement of CD22-associated PTP detection.

Results

Redistribution of CD45 leads to alterations in CD22 phosphorylation and effector protein recruitment

Previous studies have demonstrated that CD22, CD45, and the BCR physically interact with one another in the plasma membrane (25–28), suggesting that a functional relationship may exist among these proteins. That this is true for the BCR and CD22 has been demonstrated by several laboratories in experiments showing that cross-linking of the BCR leads to increased tyrosine phosphorylation of CD22 (29, 30). Thus, it was of interest to determine whether perturbation of CD45 leads to alterations in the phosphorylation status of CD22 as well. Previous studies have been performed demonstrating that redistribution of CD45 within the plasma membrane of B cells can lead to alterations in the phosphorylation of other transmembrane proteins such as Igα and Igβ (31).

FIGURE 1. CD22 and CD45 do not colocalize in the plasma membrane in response to CD45 cross-linking. Cells (1 × 10^6) were washed in PBS with Ca$^{2+}$ and were incubated in the presence of anti-CD45 mAb (biotinylated 13/2.3, 22 μg/ml) for 20 min on ice. These cells were washed in PBS with Ca$^{2+}$ and were incubated in PBS with Ca$^{2+}$ alone (A) or in the presence of a secondary cross-linking reagent (B; FITC-conjugated streptavidin, 5 μg/ml) for 30 min at 37°C. After stimulation cells were washed in PBS without Ca$^{2+}$ and Mg$^{2+}$ containing 2% sodium azide and 2% FBS and were incubated with anti-CD22 mAb (PE-conjugated NIMR-6) for 20 min on ice. Slides were prepared by cyto-spinning the cells onto glass slides, after which they were incubated in ice-cold ethanol overnight. Fixed slides were washed and mounted in Fluoromount G and viewed with a Leica/Leitz DMRB fluorescence microscope equipped with appropriate filter cubes.

Experiments to measure CD22-associated protein tyrosine phosphatase activity were performed using Tyrosine Phosphatase Assay Kit I from Upstate Biotechnology following the manufacturer’s protocol. The samples were prepared for assay by stimulating K46 cells, 35S5 CD45-negative variants, or 35S5:SHP-1:C453S transfectants (2 × 10^7/sample) with mAbs directed against the BCR as described previously. The cells were washed with PBS and lysed on ice in 0.5 ml of buffer containing 1% Nonidet P-40, 0.5% SDS, and 1 mM DTT. The lysates were centrifuged and precleared, and CD22 was immunoprecipitated as described above. Immune complex-coated beads were washed once in wash buffer (50 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.1% Nonidet P-40), twice in WG buffer (50

Measurement of calcium mobilization

Studies were performed with K46 cells, the 35S5 clone, 35S5 vector control transfectants, and 35S5:SHP-1:C453S transfectants in which Ca$^{2+}$ mobilization was assayed in response to BCR or MHC class II cross-linking as described previously (6). Cells were loaded with the Ca$^{2+}$ indicator dye indo-1/AM (Molecular Probes, Eugene, OR) at a final concentration of 5 μM. Cells loaded with indo-1 were analyzed using a Becton Dickinson FacsVantage flow cytometer (San Jose, CA) equipped with an Enterprise laser from Coherent (Santa Clara, CA) set for excitation at approximately 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 bandpass filters to detect violet and blue, respectively. The ratio of emissions was calculated, and a plot was constructed of fluorescence ratio vs time. Analysis of BCR- and MHC class II-mediated Ca$^{2+}$ mobilization was performed as previously described (6). For class II Ca$^{2+}$ response measurements, the cells were incubated with biotinylated anti-class II mAb (D3.137.5.7, 10 μg/ml, 15 min at room temperature) and washed, and an unstimmulated baseline was established. Once a baseline measurement for intracellular free Ca$^{2+}$ was recorded, streptavidin (10 μg/ml) was added, and the analysis was resumed. The BCR-mediated Ca$^{2+}$ mobilization response was initiated by the addition of anti-μ (B76, 10 μg/ml) once a baseline was established. Measurement of the intracellular vs extracellular influx of Ca$^{2+}$ was performed as previously described (6). Cells were resuspended in Ca$^{2+}$- and Mg$^{2+}$-free-PBS just before analysis, and a baseline was established. The cells were stimulated as described above with mAb directed against the BCR or MHC class II, and the resulting Ca$^{2+}$ mobilization response was allowed to return to baseline. Once the baseline was resolved, CaCl$_2$ was added to a final concentration of 4 mM, and measurement of extracellular Ca$^{2+}$ influx was initiated.
CD22 is hyperphosphorylated in the absence of CD45, resulting in enhanced recruitment of SHP-1.

To further examine the potential role of CD45 in regulating tyrosine phosphorylation of CD22, experiments were performed using CD45-deficient variants of the K46 cell line. These CD45-deficient cells were generated by chemical mutagenesis and have been characterized previously (6). K46 parental cells and the CD45-negative 35S5 mutant were stimulated through the BCR in an analogous manner (6). Thereafter, experiments were performed to evaluate whether cross-linking of MHC class II molecules and the BCR in an analogous manner (6).

As depicted in Fig. 4, SHP-1 isolated from 35S5 cells exhibited an 8- to 10-fold increase in its binding to a tyrosine-phosphorylated protein migrating at 140 kDa compared with SHP-1 isolated from K46 parental cells. When the membrane was stripped and reprobed with Ab directed against SHP-1, it was noted that the amount of SHP-1 in each lane was comparable (Fig. 4).

Studies have shown that CD45 regulates signaling via MHC class II molecules and the BCR in an analogous manner (6). Therefore, experiments were performed to evaluate whether cross-linking of class II leads to hyperphosphorylation of CD22 in the absence of CD45. Cross-linking of MHC class II was observed to induce phosphorylation of CD22 regardless of whether CD45 was present (Fig. 5). As seen previously, the CD45-negative cells exhibited both basal and inducible hyperphosphorylation of CD22 compared with CD45-positive K46 cells. When the membrane was stripped and reprobed to detect SHP-1, the amount of SHP-1 bound to CD22 was several times greater (≥10-fold) in the CD45-negative cells than in the K46 parental cells. Thus, CD45 plays a comparable role in regulating CD22 phosphorylation via BCR- and MHC class II-dependent signaling pathways in the B cell.
Lyn associates with CD22 in CD45-deficient cells

Expression of the Src family kinase Lyn has recently been shown to be essential for inducible tyrosine phosphorylation of CD22 in response to BCR cross-linking (17–20). Moreover, previous studies have demonstrated that CD45 expression is required for activation of the Src family kinases, including Lyn (6, 8, 10). We have shown that in the CD45-deficient 35S5 cell line, activation of Lyn is attenuated in response to BCR or class II cross-linking (6). Because of the functional relationship among CD45, Lyn, and CD22, the finding that BCR or class II cross-linking leads to hyperphosphorylation of CD22 in the absence of CD45 expression was not expected. It was therefore of interest to further examine the relationship between CD22 and Lyn in the 35S5 cell line. It has been shown that Lyn is recruited to CD22 in response to stimulation through the BCR (32, 33). The nature of the physical interaction between Lyn and CD22 has yet to be fully elucidated, although it may be mediated at least in part by a phosphotyrosine-independent mechanism (33).

Experiments were conducted in which K46 and 35S5 cells were stimulated with anti-IgM (B76) mAb followed by immunoprecipitation of CD22 from detergent-solubilized lysates. Western blot analysis to measure tyrosine phosphorylation revealed inducible activation of Lyn in unstimulated cells and at early time points (1–10 min) in the 35S5 cell line, compared with that from K46 parental cells, as described above (data not shown). When the CD45-negative clone 35S5 (1×10^7 cells/sample) in which cells were incubated in medium alone (NT) or in the presence of biotinylated anti-class II mAb (D3.137.5.7, 10 µg/ml) for 15 min at room temperature. Cells were then washed in PBS, resuspended in IMDM with 5% FCS, and incubated for various times with streptavidin at a final concentration of 10 µg/ml. At the appropriate time points, cells were resuspended in ice-cold PBS, washed, and lysed in buffer with 1% Nonidet P-40, and CD22 was immunoprecipitated as previously indicated. Tyrosine phosphorylation of CD22 (top panel) and SHP-1 recruitment (lower panel) were visualized as described previously.

Overall activation of Lyn may be attenuated in the absence of CD45, its association with CD22 in the 35S5 cells is unaffected, thereby providing a potential explanation for the increased basal and inducible levels of CD22 tyrosine phosphorylation.

Expression of catalytically inactive SHP-1 restores the intracellular Ca^{2+} mobilization response in CD45-deficient cells

Previous studies have shown that the loss of CD45 expression has a significant effect on the BCR-mediated Ca^{2+} mobilization.

CD45 expression is not required for the physical association between CD22 and the PTK Lyn. K46 and 35S5 (1×10^7/sample) cells were incubated in medium alone (NT) or in the presence of anti-IgM mAb (B76, 10 µg/ml) for the amount of time indicated. mAb-mediated stimulation of B cells was stopped by the addition of ice-cold PBS. The cells were washed and resuspended in buffer containing 1% Nonidet P-40 for 1 h on ice. The lysates were precleared, and CD22 was immunoprecipitated using the mAb NIMR-6 coupled to Sepharose 4B. Immune complexes were resolved by SDS-PAGE on 10% acrylamide gels, and the proteins were transferred to nitrocellulose. The association of Lyn was detected using a polyclonal rabbit anti-mouse Lyn Ab followed by the addition of a secondary goat anti-rabbit Ig Ab coupled to HRPO. The Lyn band was visualized using ECL.
response in B cells (6, 34). The 35S5 CD45-negative cell line exhibits little or no extracellular influx of Ca\(^{2+}\) in response to BCR cross-linking, whereas the mobilization of Ca\(^{2+}\) from intracellular stores is relatively unaffected (6). In contrast, a more pronounced effect on class II signaling is observed in which both components of the Ca\(^{2+}\) mobilization response are significantly attenuated (6). Because CD22 was observed to be hyperphosphorylated in the 35S5 CD45-negative cell line, leading to enhanced recruitment of SHP-1, experiments were performed to determine whether the aberrant recruitment of SHP-1 is responsible for attenuation of signal transduction via the BCR and MHC class II activation complexes.

Catalytically inactive SHP-1 with a C453S mutation (SHP-1: C453S) was transfected into 35S5 cells. The C453S mutation in SHP-1 inhibits its ability to catalyze the dephosphorylation of substrates, but not its ability to bind to them (35). To identify transfectants that expressed the catalytically inactive SHP-1:C453S mutant, lysates from 35S5 cells transfected with vector alone and with vector containing the SHP-1:C453S cDNA were incubated with anti-Myc mAb, and the immune complex material was subjected to immunoblot analysis to determine the levels of Myc-tagged SHP-1:C453S expressed in the cells. As depicted in Fig. 7A, neither the 35S5 nor the 35S5 vector control cells expressed Myc-tagged SHP-1:C453S, whereas 35S5 cells transfected with the SHP-1: C453S cDNA expressed significant amounts of the catalytically inactive PTP. To further analyze SHP-1: C453S transfectants, experiments were performed to assay binding of Myc-tagged SHP-1: C453S to CD22. As depicted in Fig. 7B, when the 35S5:SHP-1: C453S cells were stimulated through the BCR, and CD22 was immunoprecipitated, immunoblotting for Myc-tagged SHP-1: C453S demonstrated that the catalytically inactive PTP binds to CD22. Additionally, the association between Myc-tagged SHP-1: C453S and CD22 appeared to be inducible, although it should be noted that significant binding of SHP-1: C453S to CD22 could be detected in unstimulated cells.

Subsequent experiments were performed to measure the relative levels of PTP activity associated with CD22 in K46, 35S5, and 35S5:SHP-1: C453S cell lines. CD22 was immunoprecipitated from unstimulated and anti-Ig-treated cell lines, and the PTP activity associated with it was assayed as described in Materials and Methods. As expected, CD22 isolated from the unstimulated 35S5 cell line was associated with a 2- to 3-fold increase in the amount of PTP activity compared with that in K46 parental cells. After stimulation with anti-Ig, the amount of PTP activity associated with CD22 isolated from 35S5 cells was 10-fold greater. In contrast, the amount of PTP activity associated with CD22 isolated from anti-Ig-treated 35S5:SHP-1: C453S transfectants was 1.5- to 2-fold above that seen for the K46 parental cells. These data indicated that catalytically inactive SHP-1: C453S was effectively competing with endogenous SHP-1 for binding sites on CD22, thereby decreasing the total PTP activity associated with CD22 in the CD45-negative cells. These experiments confirmed that the 35S5:SHP-1: C453S transfectant cell line was an appropriate system in which to analyze whether aberrant recruitment of SHP-1 to CD22 results in attenuation of signal transduction.

Loss of CD45 expression has a more profound effect on the Ca\(^{2+}\) mobilization response associated with cross-linking of MHC class II. Therefore, studies were performed to determine whether expression of the SHP-1: C453S mutant restores signaling via MHC class II in CD45-deficient B cells. Analysis of Ca\(^{2+}\) mobilization in response to class II cross-linking revealed that the response is almost completely abrogated in the absence of CD45 (Fig. 8A, compare K46 vs 35S5 cells). Expression of the SHP-1: C453S mutant, however, partially restores the MHC class II-mediated Ca\(^{2+}\) response in the absence of CD45 (Fig. 8A, compare K46 with 35S5:SHP-1: C453S). As can be seen, the response in the SHP-1: C453S transfectants exhibits a transient phenotype characteristic of Ca\(^{2+}\) mobilization from intracellular stores without subsequent influx of Ca\(^{2+}\) from the extracellular space. This was confirmed by performing the assay in the presence of EGTA (data not shown). Because signaling through the BCR leads to mobilization of Ca\(^{2+}\) from intracellular stores even in the absence of CD45, it was of interest to determine whether the SHP-1: C453S mutant could restore the extracellular influx component of the BCR response in CD45-deficient cells. As depicted in Fig. 8B, expression of catalytically inactive SHP-1: C453S does not significantly alter the BCR-mediated Ca\(^{2+}\) mobilization response in cells lacking
CD45. These findings suggest that aberrant recruitment and activation of SHP-1 in CD45-deficient cells may be involved in regulating mobilization of Ca\textsuperscript{2+} from intracellular stores in response to class II cross-linking. K46 parental cells, 35S5, 35S5: vector control (VC), and 35S5: SHP-1: C453S transfectants (1 × 10\textsuperscript{6} cells/sample) were loaded with indo-1 as described in Materials and Methods. Experiments were then performed in which Ca\textsuperscript{2+} mobilization was assayed in response to stimulation through MHC class II (A; 10 μg/ml biotinylated D3.137 and 10 μg/ml streptavidin) or the BCR (B; B7.6, 10 μg/ml). For the analysis of class II-mediated Ca\textsuperscript{2+} mobilization, cells were incubated with anti-class II mAb for 10 min at room temperature. The cells were washed, and the Ca\textsuperscript{2+} baseline was established. The analysis was stopped, and streptavidin was added, after which the analysis was resumed immediately. The response to BCR cross-linking was measured by establishing an unstimulated baseline after which the analysis was stopped, and anti-Ig mAb was added. The analysis was reintiated immediately after the addition of anti-Ig mAb. The Ca\textsuperscript{2+} ionophore ionomycin was used to ensure that cells were loaded equivalently with indo-1 (data not shown).

Discussion

Previous studies have presented findings that support the hypothesis that CD45 and CD22 exist in the B cell plasma membrane as part of a multimolecular complex (24–28, 32, 35, 36). The results presented in the current study further indicate that a functional relationship exists between CD45 and CD22 based on the observation that CD45 is involved in regulating the tyrosine phosphorylation status of CD22. This conclusion is based on two experimental approaches. First, it was shown that mAb-mediated redistribution of CD45 in the plasma membrane is associated with enhanced tyrosine phosphorylation of CD22. Whether cross-linking of CD45 simply restricts its ability to physically engage substrates in the cell (e.g., CD22), results in inhibition of its catalytic function due to dimerization, or both is not known. Previous studies have suggested that CD45 cross-linking and physical sequestration in the membrane lead to enhanced tyrosine phosphorylation of putative substrates without affecting CD45 catalytic activity (31). Alternatively, studies have shown that ligand binding to an EGF:CD45 chimeric protein leads to dimerization of chimeric CD45 and inhibition of its catalytic function due to the reciprocal insertion of a wedge domain from one polypeptide into the PTP active site of the other, thereby preventing substrate binding (37, 38). Clearly, the processes of physical sequestration and inhibition of catalytic activity are not mutually exclusive and may both be involved in regulating CD45 function in vivo.

A second approach used in the current study to demonstrate that CD45 is involved in regulating CD22 phosphorylation was to compare the basal and inducible tyrosine phosphorylation of CD22 isolated from CD45-positive (K46) and CD45-deficient (35S5) B cell lines. The results obtained demonstrate that both basal and inducible phosphorylation of CD22 are increased in cells that lack CD45. These findings corroborate the results from the CD45 cross-linking experiments. Nevertheless, neither approach conclusively proves that CD22 is a direct substrate for CD45. It is equally likely that cross-linking of CD45 or elimination of its expression alters the activity of other PTPs or PTKs and that perturbation of the normal homeostasis of reversible tyrosine phosphorylation indirectly leads to increased phosphorylation of CD22.

Previous studies have demonstrated that the PTK Lyn plays a critical role in mediating inducible tyrosine phosphorylation of CD22 (17–20). It was therefore surprising to find that CD22 is hyperphosphorylated in CD45-deficient B cells, indicating that Lyn is able to function in the absence of CD45 with respect to CD22 phosphorylation. Because studies have demonstrated that Lyn binds to CD22 via an undefined mechanism, and that Lyn can be recruited to CD22 in an inducible manner in response to BCR cross-linking (32, 33), it was hypothesized that this interaction is important for Lyn-dependent phosphorylation of CD22. Indeed, Lyn was observed to coprecipitate with CD22 regardless of CD45 expression. Moreover, Lyn appeared to be constitutively associated with CD22, and the amount of Lyn bound to CD22 was elevated in the absence of CD45 compared with that in K46 parental cells. The mechanism responsible for enhanced recruitment of Lyn in the absence of CD45 expression is not known at present. Although previous studies indicate that the association may not be entirely dependent on tyrosine phosphorylation of CD22 (33), suggesting that Lyn may interact with CD22 via residues in its unique amino-terminal region. This could facilitate Lyn-dependent basal phosphorylation of CD22, leading to hyperphosphorylation in the absence of CD45 and increased recruitment of Lyn via a phosphotyrosine-dependent binding process.

Binding of Lyn to CD22 via a phosphotyrosine:SH2 domain interaction could promote Lyn activation even though CD45 is not present. It is formally possible that tyrosine phosphorylation of CD22 generates phosphotyrosine motifs in its cytoplasmic tail that bind to the SH2 domain of Lyn with high affinity such that they effectively compete with the carboxyl-terminal phosphotyrosine residue in Lyn. Displacement of the Lyn carboxyl-terminal inhibitory phosphotyrosine residue by binding of its SH2 domain to one or more phosphotyrosine motifs in the cytoplasmic tail of CD22 would be predicted to derepress the enzymatic activity of Lyn. Thus, even though the overall level of Lyn activity may be decreased in CD45-deficient cells, the catalytic activity of Lyn associated with CD22 would be unaffected. An alternative explanation is that Lyn could act as an intermediate linker protein that facilitates the recruitment of another PTK that actually phosphorylates CD22. In this scenario Lyn activation would not be essential...
for CD22 phosphorylation, whereas its physical association with CD22 would. It is interesting to note that none of the studies examining Lyn knockout mice has definitively proven that Lyn phospholates CD22. These studies have correlated Lyn expression with CD22 phosphorylation, which proves that Lyn has to be present in the cell, although they have not demonstrated that Lyn itself phosphorylates CD22.

Hyperphosphorylation of CD22 in CD45-deficient B cells was observed to correlate with enhanced recruitment of the PTP SHP-1. Because the 35S5 CD45-deficient B cell line has been shown to exhibit decreased Ca²⁺ mobilization in response to BCR and MHC class II cross-linking (6), it was of interest to determine whether this was due to aberrant recruitment of SHP-1 to CD22. To address this question a catalytically inactive form of SHP-1 with a C453S point mutation was expressed in the 35S5 cell line to compete with endogenous wild-type SHP-1 for binding to phosphorylated CD22. Indeed, it was observed that the SHP-1 C453S mutant was able to compete with endogenous SHP-1 for binding to CD22, thereby restoring the associated PTP activity to a level comparable to that observed in CD45-positive cells. Nevertheless, catalytically inactive SHP-1 did not fully restore the Ca²⁺ mobilization response elicited by cross-linking of either the BCR or MHC class II. Although intracellular mobilization of Ca²⁺ was potentiated by expression of the C453S SHP-1 mutant, particularly in response to class II cross-linking, the influx of Ca²⁺ from the extracellular space was not restored regardless of the stimulus.

Previous studies have demonstrated that crossing CD45 knock-out mice with the motheaten viable strain, which expresses a mutant form of SHP1 that is catalytically defective, corrects developmental abnormalities associated with the loss of either PTP alone (39). Moreover, functional elimination of both PTPs was observed to restore BCR-mediated signal transduction processes such as PTK and MAP kinase activation. These data suggest that CD45 and SHP-1 are functionally interrelated and that they act in a coordinate manner to regulate BCR signaling, leading to activation and maturation. The results presented in the current study support the concept that CD45 and SHP-1 functionally counterbalance one another to regulate specific signal transduction processes. However, it is unlikely that these PTPs have completely overlapping regulatory functions because expression of catalytically inactive SHP-1 fails to restore long term, receptor-mediated influx of Ca²⁺ from the extracellular space.

Based on the results obtained, it is likely that CD45 regulates distinct signaling components that control Ca²⁺ influx. The Ca²⁺ response seen in the CD45-deficient 35S5 cells is similar to that observed when the FcγRIIb receptor is cotigated with the BCR (40, 41). This raises the possibility that loss of CD45 expression leads to dysregulation of SH2-containing inositol 5'-phosphatase (SHIP) function that could, in turn, affect phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-P3) production and BTK activation. This raises the possibility that loss of CD45 expression or indirectly through regulation of PTK/PTP activity in the B cell. Further elucidation of the specific nature of the defect in Ca²⁺ mobilization is of interest because it will provide important information concerning the mechanism(s) by which CD45 regulates transcription factor activation and thus the threshold of signaling through the BCR.

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