The Motheaten Mutation Rescues B Cell Signaling and Development in CD45-deficient Mice

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Summary

The cytosolic SHP-1 and transmembrane CD45 protein tyrosine phosphatases (PTP) play critical roles in regulating signal transduction via the B cell antigen receptor (BCR). These PTPs differ, however, in their effects on BCR function. For example, BCR-mediated mitogenesis is essentially ablated in mice lacking CD45 ($CD45^-$), but is enhanced in SHP-1–deficient motheaten (*me*) and viable motheaten (me^v) mice. To determine whether these PTPs act independently or coordinately in modulating the physiologic outcome of BCR engagement, we assessed B cell development and signaling in CD45-deficient me^v ($CD45^-/SHP\text{-}1^-$) mice. Here we report that the $CD45^-/SHP\text{-}1^-$ cells undergo appropriate induction of protein kinase activity, mitogen-activated protein kinase activation, and proliferative responses after BCR aggregation. However, BCR-elicited increases in the tyrosine phosphorylation of several SHP-1–associated phosphoproteins, including CD19, were substantially enhanced in $CD45^-/SHP\text{-}1^-$, compared to wild-type and $CD45^-$ cells. In addition, we observed that the patterns of cell surface expression of μ, δ, and CD5, which distinguish the PTP-deficient from normal mice, are largely restored to normal levels in the double mutant animals. These findings indicate a critical role for the balance of SHP-1 and CD45 activities in determining the outcome of BCR stimulation and suggest that these PTPs act in a coordinate fashion to couple antigen receptor engagement to B cell activation and maturation.

The pivotal role for B cell antigen receptor (BCR)[1] stimulation in driving B lymphocyte differentiation and activation is realized through a complex intracellular signaling network that biochemically translates BCR engagement to nuclear response. Transmission of ligand binding signals via this biochemical network is dependent upon reversible protein tyrosine phosphorylation and mediated by the relative effects of protein tyrosine kinases (PTKs) and phosphatases (PTPs; 1, 2). As the BCR lacks intrinsic tyrosine kinase activity, tyrosine phosphorylation of its Ig α and β chains after ligand engagement is achieved through recruitment of cytosolic PTKs, the activities of which create phosphotyrosine sites for recruitment and activation of SH2 domain containing PTKs and other secondary signaling molecules (3). PTK-induced phosphorylation thus provides the framework for the sequential protein activation and amalgamation that ultimately serves to couple BCR stimulation to lymphocyte response.

At present, the regulatory roles for PTPs in BCR signaling are not as well defined as those of PTKs. However, two PTPs that have been identified as key elements in modulating the outcome of BCR engagement are the CD45 transmembrane and SHP-1 cytosolic proteins, enzymes that are both expressed in hemopoietic cell lineages (4, 5). Analyses of CD45-deficient mutant cell lines as well as B cells from mice genetically deficient for CD45 have indicated that CD45 activity is used to couple BCR stimulation to cell proliferation (6–8). The involvement of CD45 in B cell differentiation has also been revealed by the recent findings that CD45-deficient mice manifest a reduction in splenic B cells with phenotypic markings of the mature B cell pool (8). Together, these data suggest a critical role for CD45 in promoting the coupling of BCR stimulation to both B cell mitogenesis and transit from the immature to mature stage of differentiation. Similarly, multiple lines of evidence indicate that the SH2 domain–containing SHP-1 tyrosine phosphatase plays a major role in the regulation of BCR signaling capacity. These data include, for example, the demonstration that loss of function mutations in the SHP-1

[1]*Abbreviations used in this paper:* BCR, B cell antigen receptor; HEL, hen egg lysozyme; HRP, horseradish peroxidase; MAP, mitogen-activated protein; *me*, motheaten; me^v, viable motheaten; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphase.

gene are responsible for the severe haemopoietic abnormalities found in motheaten (*me*) and viable motheaten (me^v) mice (9, 10). These animals, which express no (*me*) or catalytically compromised (me^v) SHP-1, manifest high levels of serum immunoglobulins and autoantibodies. In addition, they exhibit a profound reduction in conventional B-2 cells, but an overexpansion of $CD5^+$ B-1 cells in the periphery (11, 12). B cells from *me* and me^v mice have also been shown to be hyperresponsive to BCR stimulation, the mutant cells proliferating in response to normally submitogenic concentrations of F(ab′)2 anti-Ig antibody, but responding normally to other mitogenic stimuli such as LPS (13). Developing B cells from me^v mice bearing hen egg lysozyme (HEL) and anti-HEL transgenes have also been shown to be hyperresponsive to HEL stimulation, the anti-HEL–bearing SHP-1–deficient cells undergoing deletion when exposed to a level of antigen below that normally required to induce deletion in this system (14). Together, these data indicate a major role for SHP-1 in modulating B cell development and in regulating the signaling events linking the BCR to both proliferation and clonal deletion/negative selection. In contrast to CD45, however, SHP-1 effects on BCR signaling appear largely inhibitory, a contention also consistent with recent data indicating that SHP-1 interacts with and modulates the signaling functions of both the FcγRIIB1 and CD22 receptors, two transmembrane molecules also implicated in the downregulation of BCR-elicited signaling cascades (15–18).

Although the available data indicate opposing effects of CD45 and SHP-1 on the signaling events triggered by BCR engagement, it is currently unclear whether these PTPs exert their antagonistic effects by coordinate regulation of a single signaling pathway or by the modulation of distinct, parallel signaling cascades involving disparate downstream signaling effectors. It is also unclear whether the effects of these individual PTPs on B cell maturation are realized via the modulation of BCR signaling capacity and, in particular, through the alteration of BCR thresholds for signal propagation to the nucleus. To address these issues, we have examined the ontogeny and signaling properties of the B lineage population that develops in mice lacking both the CD45 and SHP-1 tyrosine phosphatases. Analysis of these mice has revealed their expression of a peripheral B cell population comprised largely of mature, conventional B-2 cells that proliferate in response to BCR engagement. As described herein, these data strongly suggest that SHP-1 and CD45 act in concert to modulate the coupling of BCR stimulation to mitogenesis and maturation.

Materials and Methods

Cell Preparation and Proliferation Analysis. Single cell suspensions of splenic B cells were obtained from the PTP-deficient control mice after previously published procedures (8, 13). For FACS® analysis, the spleen cells were subjected to erythrocyte lysis in ammonium chloride. Cells ($1–5 \times 10^5$/sample) were stained by standard procedures using FITC–anti-IgM (hybridoma 33-60), PE–anti-IgD (Southern Biotechnology Assoc., Birmingham, AL), and PE–anti-CD5 (PharMingen, San Diego, CA) antibodies and 10^4 cells/sample were analyzed on a FACScan® flow cytometer (Becton Dickinson, San Jose, CA). For proliferation assays, single cell suspensions of splenic cells were subjected to erythrocyte lysis in ammonium chloride, T cell lysis by anti-CD4/CD8/Thyl.2 antibody and complement treatment, and separation over Percoll gradients as previously described (8, 13). The purified splenic B cells (>80% sIg^+ by FACS®) were cultured (5×10^4 cells/well) for 48 h in culture media alone or in the presence of 5 μg/ml B76 (rat IgG1 anti–mouse IgM) or 15 μg/ml LPS, and proliferation was evaluated after a 6-h pulse with 1 μCi/well [^{3}H]thymidine (Dupont/New England Nuclear, Boston, MA). Data are expressed as cpm and values represent means (± SEM) of triplicate experiments.

Mouse Strain Typing. Genotyping for the me^v mutation was carried out by PCR amplifying a 69-bp fragment encompassing the site of the T→A transversion in the me^v SHP-1 gene using the primer pair 5′-CGTGTCATCGTCATGACT-3′ (forward) and 5′-AGGAAGTTGGGGCTTTGCCGT-3′ (back primer that introduces an RsaI site in the vicinity of the mutation) followed by RsaI digestion of the PCR products. The wild-type SHP-1 allele is detected as 48- and 21-bp RsaI restriction fragments, whereas the me^v allele shows as an intact 69-bp fragment. The genotype with respect to CD45 was determined by cell surface analysis of the B220 isoform of CD45 since, as previously described, there is complete correlation between the presence of B220 and the presence of the wild-type CD45 gene (19). The $CD45^-$ mice used in these studies were from the eighth backcross generation to C57BL/6.

Antitopoisomerase Assay. Serial dilutions of sera in PBS/3% FCS were added to topoisomerase coated wells (Scl-70; Advanced Biological Products, Brampton, Canada) and incubated for 1 h at room temperature. Wells were washed, incubated with goat anti–mouse Ig coupled to horseradish peroxidase (HRP; Sigma Chemical Co., St. Louis, MO) for 1 h, washed again, incubated with substrate (2,2′-azino-bis; Sigma Chemical Co.), and absorbance read at 405/630 nm. To quantify the amount of anti-topoisomerase, serially diluted purified mouse IgG was added to ELISA plates (Costar Corp., Cambridge, MA) coated with goat anti–mouse IgG (Sigma Chemical Co.), and the plates were then washed, blocked with PBS/3% FCS, and developed as described above. Based on the standard, OD values of 70, 50, and 30 were estimated to correspond to ~30, 15, and 1 ng/ml, respectively.

Histological Analysis. After fixation in formalin and paraffin embedding, 3-micron sections of renal tissues harvested from normal and mutant mice were successively incubated at room temperature with rabbit anti–mouse IgM (1.5 h) and biotinylated goat anti–rabbit (30 min) antibodies (Zymed Labs., Inc., S. San Francisco, CA), peroxidase-conjugated streptavidin (30 min), and aminoethylcarbazole in 0.2 M sodium acetate (15 min; Sigma Chemical Co.). Sections were then counterstained in hematoxylin and mounted with Crystal/Mount (Biomeda Corp., Foster City, CA). Original magnification was at 250. Positive staining is indicated by the red-brown deposition seen most prominently in the lower left panel (Fig. 2 *C*).

Immunoprecipitation and Immunoblotting. Before preparation of cell lysates, purified splenic B cells were resuspended in culture media at 3×10^7/ml and left untreated or, alternatively, stimulated for 5 min at 37°C with 5 μg/ml B76 anti-Ig antibody. For biotinylation, $5–6 \times 10^7$ splenic B cells were resuspended in ice-cold 1.5 ml PBS and mixed with 15 μl sulfo-NHS-Biotin solution (Pierce Chem. Co., Rockford, IL). After 30 min of incuba-

tion at room temperature, the reaction was quenched by 5 min of incubation with 500 μg/ml lysine in PBS. Cells were then stimulated as above. For preparation of cell lysates from nonbiotinylated or biotinylated cells, the cells were pelleted by 0.5 min centrifugation and lysed for 15 min in 400 μl cold buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 8, 2 mM EDTA, 50 mM NaF, 1 mM PMSF, 1 mM sodium orthovanadate, 0.05% NaN_3, and 1% NP-40. Lysates were then centrifuged at 4°C for 10 min at 14,000 *g*, electrophoresed through SDS-polyacrylamide gels, transferred to nitrocellulose, and incubated at 4°C for at least 1 h in Tris-buffered saline with Tween solution (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 0.05% Tween 20) plus 3% gelatin. Filters were then incubated for 2 h at room temperature with antiphosphotyrosine antibody (4G10; Upstate Biotechnology Inc., Lake Placid, NY) in Tris-buffered saline with Tween followed by the space addition of goat anti–mouse antiserum labeled with peroxidase (Amersham Corp., Arlington Heights, IL) and HRP conjugate (Bio Rad Labs., Hercules, CA). Filters derived using protein lysates from biotinylated cells were also probed with HRP-avidin (Pierce Chem. Co.). Immune complexes were detected using an enhanced chemiluminescence system (Amersham Corp.). For immunoprecipitations, lysate proteins (1 mg) were precleared by incubation with protein A–Sepharose (Pharmacia, Baie d'Urfe, Quebec; 40 μl lysate in 1 ml volume beads) for 1 h at 4°C and for an additional hour with 40 μl beads and 5 μl rabbit preimmune serum. Lysates were then incubated for 3 h at 4°C with 5 μl anti–SHP-1 (rabbit polyclonal produced in our lab) or anti-CD19 (rat monoclonal produced by the ID3 hybridoma) antibodies or rabbit preimmune serum and 10 μl packed protein A–Sepharose beads and the immune complexes then collected by centrifugation, washed in lysis buffer, and resuspended in SDS sample buffer. Samples were then boiled for 5 min, electrophoresed through 8% SDS-PAGE, and subjected to immunoblotting analysis as described above. Stripping and reprobing of the blots were performed according to Amersham Corp.'s recommended protocol. Antibody to mb-1 (rabbit anti–mb-1 cytoplasmic tail) was provided by Dr. L. Matsuuchi (University of British Columbia, Vancouver, Canada).

Assay of Mitogen-activated Protein Kinase Activity. Cell lysates were prepared from unstimulated and anti-Ig antibody (5 μg/ml B76)–treated splenic B cells (3×10^7) from the various PTP-deficient mice and the lysate proteins then immunoprecipitated with anti-Erk2 antibody (Santa Cruz Labs., Santa Cruz, CA). Equal aliquots of the anti-Erk2 immune complexes were then resuspended in 25 μl reaction buffer (30 mM Tris-HCl, pH 8.0, 20 mM $MgCl_2$, 2 mM $MnCl_2$ containing 5 μg myelin basic protein (Upstate Biotechnology Inc.) and 10 μg [γ^{32}P]ATP (Dupont/New England Nuclear). After a 15 min incubation at 30°C, reactions were terminated by addition of 6 μl 5× loading buffer and the samples were then boiled, electrophoresed through 15% polyacrylamide gels, and the phosphorylated myelin basic protein bands were visualized by phosphoimaging. Equal aliquots of the remaining portions of the anti-Erk2 immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, and the filters were then probed as described above with anti-Erk 2 antibody.

Results and Discussion

To address the relative contribution of the CD45 and SHP-1 enzymes to B lymphocyte activation and development, mice lacking both SHP-1 and CD45 activities were derived by breeding the *me*^{v} mutation, which engenders expression of catalytically compromised SHP-1, into mice homozygous for a CD45–exon 6 null mutation (9, 19). Double mutant (CD45$^-$/SHP-1$^-$) mice derived from these matings developed the wasting disease normally associated with the lack of SHP-1, and consequently manifested severe runting and early mortality. However, in contrast to the splenomegaly characteristically found in the *me*^{v} homozygous (*me*^{v} or CD45$^+$/SHP-1$^-$) mice (11, 12), splenic size was markedly reduced in the double mutant animals As a consequence of these differences, double mutant littermate and age-matched wild-type control mice differed significantly with respect to splenic cellularity and composition. Thus, assessment of peripheral B cell populations in these animals requires consideration of not only absolute cell numbers, which depend highly on individual animal size, and particularly spleen size, but also evaluation of the relative frequencies of the cell populations being studied. Accordingly, to examine the effects of combined CD45/SHP-1 deficiency on B cell development, the presence of mature splenic B cells was assessed in the double mutant mice derived from these matings by evaluating the proportion as well as absolute numbers of four splenic cell populations distinguished by relative levels of cell surface μ and δ.

As shown by the representative example in Fig. 1, flow cytometric analysis of splenic cells obtained from double mutant mice (CD45$^-$/SHP-1$^-$) revealed the proportion of $\delta^{hi}\mu^{lo}$ B cells to be similar to that detected in wild-type (CD45$^+$/SHP-1$^+$) spleens (48 versus 45%). This result suggests that progression to the mature and active stage of B cell differentiation occurs despite the absence of these two PTP activities. By contrast, the proportion of $\delta^{hi}\mu^{lo}$ cells in the splenic B cell compartment of CD45$^-$ mice is markedly reduced relative to that detected in wild-type mice (7 compared to 45%). In absolute terms, the CD45$^-$ mice have threefold fewer $\delta^{hi}\mu^{lo}$ splenic B cells than do normal mice, despite having twice as many total splenic B cells. *Me*^{v} mice also have fewer $\delta^{hi}\mu^{lo}$ B cells in absolute terms, but this reduction parallels the reduced size of these animals and the reduced number of B lineage cells present. Fig. 1 also shows that two populations which emerge in *me*^{v} spleens ($\delta^-\mu^+$ and $\delta^+\mu^-$ populations) are eliminated in the normal double mutant mice. A further manifestation of impaired B cell differentiation in *me*^{v} mice is the marked over expression of CD5-expressing B-1 cells. These cells constitute the majority of splenic B cells in SHP-1$^-$ mice (see legend to Fig. 1), but represent only a minor population in the B cell compartments of either wild-type or CD45$^-$ mice. The proportion of CD5$^+$ splenic B cells is significantly reduced in double mutant CD45$^-$/SHP-1$^-$ mice, although their levels remain higher than normal indicating a failure to completely restore normal B cell development. It is possible, for example, that the B cell development in the double mutant mice is still subjected to *trans* effects mediated by abnormal monocytes. Nonetheless, these observations reveal the development of significant numbers of conventional B-2 cells in CD45$^-$/SHP-1$^-$ mice, a population that fails to develop in CD45$^+$ mice deficient for SHP-1. They

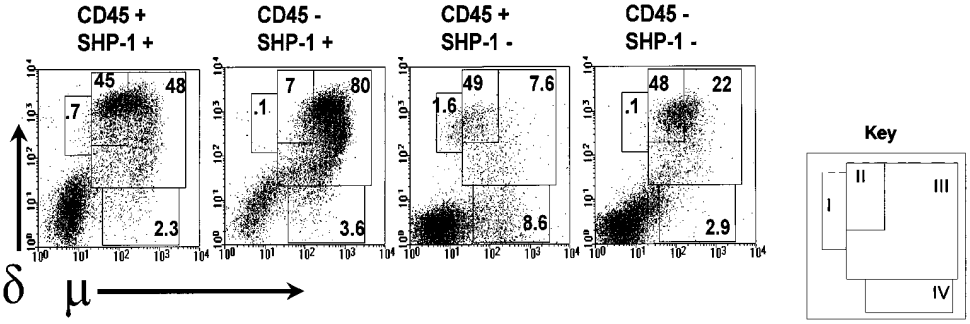

Figure 1. Representative flow cytometric analyses of splenic B cells from PTP-deficient mice. Expression of IgM and IgD was evaluated on splenic cells from age-matched 4-wk-old mice: $CD45^+/SHP\text{-}1^+$ (C57B1/6 wild type); $CD45^-/SHP\text{-}1^+$ (C57B1/6 CD45 exon $6^{-/-}$; originally derived by K. Kishihara; reference 8; eighth backcross generation), $CD45^+/SHP\text{-}1^-$ (C57B1/6 me^v/me^v); $CD45^-/SHP\text{-}1^-$ mice (derived by mating C57B1/6 CD45 exon $6^{-/-}$ [eighth backcross generation] with C57B1/6 $me^v/+$ mice). The percentages of cells within the boxed regions correspond to the following pattern: I, percent $\delta^+\mu^-$ in the lymphoid gate; II, percent $\delta^{hi}\mu^{lo}$ of III; III, percent $\delta^+\mu^+$ in the lymphoid gate; IV, percent $\delta^-\mu^+$ in the lymphoid gate. The lymphoid gate was set by standard procedures relying on the forward and side scatter properties of the splenic cells. The absolute numbers of various subsets of cells found in the spleens of the same mice ($\times 10^6$) for $CD45^+/SHP\text{-}1^+$, $CD45^-/SHP\text{-}1^+$, $CD45^+/SHP\text{-}1^-$, $CD45^-/SHP\text{-}1^-$), total splenic cells were 79, 120, 140, and 56, respectively. Lymphoid gate: 68, 95, 95, 41; $\mu^+\delta^+$: 33, 76, 7.2, 9.1; $\mu^{lo}\delta^{lo}$: 15, 5.2, 3.6, 4.4; $\mu^+\delta^-$, 1.6, 3.4, 8.2, 1.1; $\mu^-\delta^+$: 0.5, 0.06, 1.6, 0.05; μ^+CD5^+: 5.7 (representing 16% of μ^+ cells), 4.5 (6%), 11 (71%), 3.6 (37%). Data are representative of four independent experiments carried out as described in Materials and Methods.

also reveal that $CD45^-/SHP\text{-}1^-$ mice do not suffer the block in progression of B cells from the $\delta^{hi}\mu^{hi}$ to the $\delta^{hi}\mu^{lo}$ stage of differentiation that is typical of $CD45^-$ mice. The number of $\delta^{hi}\mu^{lo}$ cells that develop is appropriate for the small spleens of the double mutant mice. Together these results strongly suggest that these PTPs act in concert to promote B cell maturation and activation and specifically the differentiation events yielding B-1 or B-2 cells.

To investigate the physiologic basis for restoration of B cells displaying a normal phenotype in the $CD45^-/SHP\text{-}1^-$ mice, we examined the proliferative response of splenic B cells stimulated by BCR ligation. As assessed by [^{3}H]thymidine incorporation, anti-Ig induced proliferative responses of $CD45^-/SHP\text{-}1^-$ B cells were lower in magnitude than those observed in wild-type cells, but were substantially increased relative to the negligible response detected in similarly treated B cells from CD45-deficient mice (Fig. 2 *A*). By contrast, LPS-induced proliferation that has previously been shown to be unaffected by SHP-1 deficiency (13) was marginally enhanced in the context of CD45 deficiency, but markedly diminished in $CD45^-/SHP\text{-}1^-$ cells. These findings suggest a complex interaction between CD45 and SHP-1 in relation to the coupling of LPS stimulation to B cell proliferation, and also reveal the capacity of the me^v mutation to rescue the BCR signaling defect engendered by CD45 deficiency. Previous data have indicated that BCR-evoked proliferation is augmented in the absence of SHP-1 (13, 14). Coupled with this observation, the additional data presented here support the postulate of antigen receptor signaling thresholds (20). The threshold level required for transducing a physiologic, BCR-mediated response is presumably attained in $CD45^-/SHP\text{-}1^-$ B cells in which the inhibitory effects of SHP-1 on the BCR are eliminated, but not in $CD45^-$ B cells in which SHP-1 function is intact. These observations also suggest that the positive selection of B lymphocytes, as assessed by the progression from $\delta^{lo}\mu^{hi}$ to $\delta^{hi}\mu^{hi}$ to the $\delta^{hi}\mu^{lo}$ stage of B cell development, depends upon coordinate effects of SHP-1 and CD45 on BCR signaling capacity. The reduction of $CD5^+$ B-1 cells in $CD45^-/SHP\text{-}1^-$ mice further suggests that BCR-driven positive selection of not only mature B-2, but also B-1, cells depends on the interplay between CD45 and SHP-1. These observations are consistent with other evidence linking B-1 cell development to (auto)antigenic stimulation of the BCR (21, 22). From this perspective, the expansion of B-1 cells in SHP-1–deficient mice might be attributable to the loss of SHP-1 inhibitory effects on BCR activation and the subsequent indiscriminate triggering of unengaged BCRs. In $CD45^-/SHP\text{-}1^-$ mice, however, the absence of CD45-positive effects on BCR activation appears to counteract the heightened "excitability" of me^v BCRs so as to mitigate the preferential selection of B-1 cells. This interpretation of the data is supported by the findings of reduced splenic B-1 cell numbers in not only $CD45^-$ animals, but also Btk-deficient *xid* mice (23) as well as mice rendered genetically deficient for other signaling effectors implicated in the coupling of the BCR to cell response, such as CD19 (24) and Vav (25).

$CD45^-/SHP\text{-}1^-$ mice also exhibited reduced levels of serum immunoglobulins (data not shown) and autoantibodies, as evaluated by antitopoisomerase antibody titers (Fig. 2 *B*). Similarly, renal immune complex deposition was less evident in $CD45^-/SHP\text{-}1^-$ than in SHP-1–deficient me^v mice (Fig. 2 *C*). However, in contrast to these findings, overexpansion and tissue accumulation of myelomonocytic cells were of comparable severity in $CD45^-/SHP\text{-}1^-$ and $SHP\text{-}1^-$ mice (data not shown) and, as a result, these animals were indistinguishable in terms of their physical appearance and survival. These observations concur with previous data showing that elimination of B-1 cells by addition of the *xid* gene has no effect on me^v phenotype and mortality (26). Together, these data suggest that the impact of SHP-1 and CD45 on the signaling events that promote activation and differentiation are different in myeloid and monocytic cells than in lymphoid cells.

We next addressed the biochemical basis for the disparities observed in BCR-mediated activation events that distinguished $CD45^-/SHP\text{-}1^-$ B cells from those found in

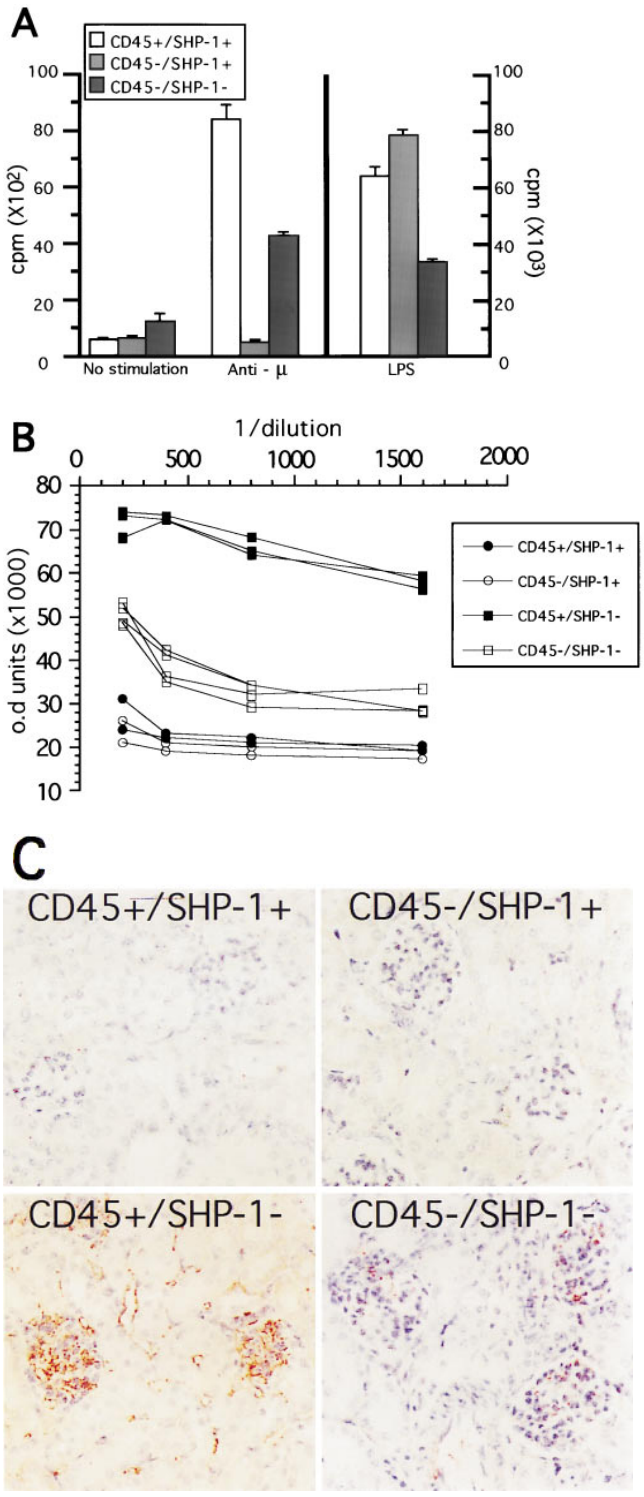

Figure 2. B cell proliferative responses and autoantibody production in PTP-deficient mice. (*A*) Effects of anti-Ig and LPS on proliferation ([^{3}H]thymidine incorporation) of CD45$^-$/SHP-1$^-$, CD45$^-$, and CD45$^+$/SHP-1$^+$ (wild-type) B cells (all LPS-treated groups as well as anti-μ–treated groups (CD45$^+$/SHP-1$^+$) and (CD45$^-$/SHP-1$^-$) differed significantly from their counterparts in the *No stimulation* group based on analysis by a two-tailed *t* test ($P < 0.05$ in all cases). (*B*) Comparisons of antitopoisomerase antibody titers in 4-wk-old CD45$^-$/SHP-1$^-$, CD45$^-$, CD45$^+$/SHP-1$^+$ and SHP-1$^-$ mice. (*C*) Immunostaining of renal tissues from 4-wk-old CD45$^+$/SHP-1$^+$, CD45$^-$, CD45$^-$/SHP-1$^-$, and SHP-1$^-$ mice.

the single PTP-deficient mice. Comparison of biochemical differences between populations of different developmental profiles should always be undertaken with caution, since the observed differences may result from a combination of direct effects due to the loss of the PTPs in question and indirect consequences that appear due to the absence of these PTPs during B cell development. Nonetheless, we compared these B cell populations by first determining their profiles of protein tyrosine phosphorylation. As is consistent with previous data linking SHP-1 to the inhibition of BCR-driven signaling cascades (13, 14) anti-Ig–induced tyrosine phosphorylation was found to be somewhat increased in the SHP-1$^-$ compared to wild-type cells (Fig. 3 *A*). By contrast, although CD45 has been implicated in PTK activation after antigen receptor engagement (6, 27), little difference was detected between stimulated CD45$^{-/-}$ and wild-type, and the CD45$^-$/SHP-1$^-$ cells with respect to the pattern or degree of total protein tyrosine phosphorylation. The capacity of SHP-1 deficiency to rescue BCR-induced proliferative responsiveness in B cells lacking CD45 suggests a role for altered tyrosine phosphorylation of proteins that normally would be dephosphorylated by SHP-1. We therefore analyzed the tyrosine phosphorylation of proteins coprecipitated with SHP-1 from these B cell populations after BCR ligation. The results of this analysis revealed the tyrosine phosphorylation of several SHP-1–associated phosphoproteins (of ~85–90, 115, and 120 kD) to be strikingly enhanced in both SHP-1$^-$ and CD45$^-$/SHP-1$^-$ cells relative to wild-type B cells. By contrast, the CD45$^{-/-}$ cells exhibited relatively reduced tyrosine phosphorylation of SHP-1 as well as the various phosphoproteins coprecipitated with this PTP, including a 140-kD species identified by immunoblotting analysis (data not shown) as CD22, a B lineage–specific transmembrane glycoprotein implicated in the negative regulation of BCR signaling (Fig. 3 *B*) (16–18, 28). In view of preliminary data from our group revealing the capacity of SHP-1 to associate with CD19, a 115–120-kD cell-specific transmembrane glycoprotein that is rapidly tyrosine phosphorylated after BCR stimulation (29, 30), the possibility that CD19 is differentially phosphorylated in the context of CD45 deficiency versus CD45/SHP-1 deficiency was directly investigated. As shown by antiphosphotyrosine immunoblotting analysis of CD19 immunoprecipitates from the various PTP-deficient B cells (Fig. 3 *C*), CD19 phosphorylation was dramatically reduced in the CD45$^-$ cells, but increased both constitutively and after BCR ligation in the CD45$^-$/SHP-1$^-$ cells compared to wild-type B cells. The observation of reduced CD19 phosphorylation in CD45$^-$ cells suggests a role for CD45 activity in promoting the tyrosine phosphorylation of this membrane glycoprotein. This may well be due to the effect of CD45 on the activation of the Lyn PTK, an enzyme previously shown to associate with CD19 (31). Conversely, the enhanced tyrosine phosphorylation of CD19 observed in SHP-1–deficient B cells (Fig. 3 *B*) suggests that SHP-1 exerts an inhibitory influence over CD19 tyrosine phosphorylation state either by direct dephosphorylation of this protein or by negative regulation of a PTK involved in CD19 phosphorylation. This

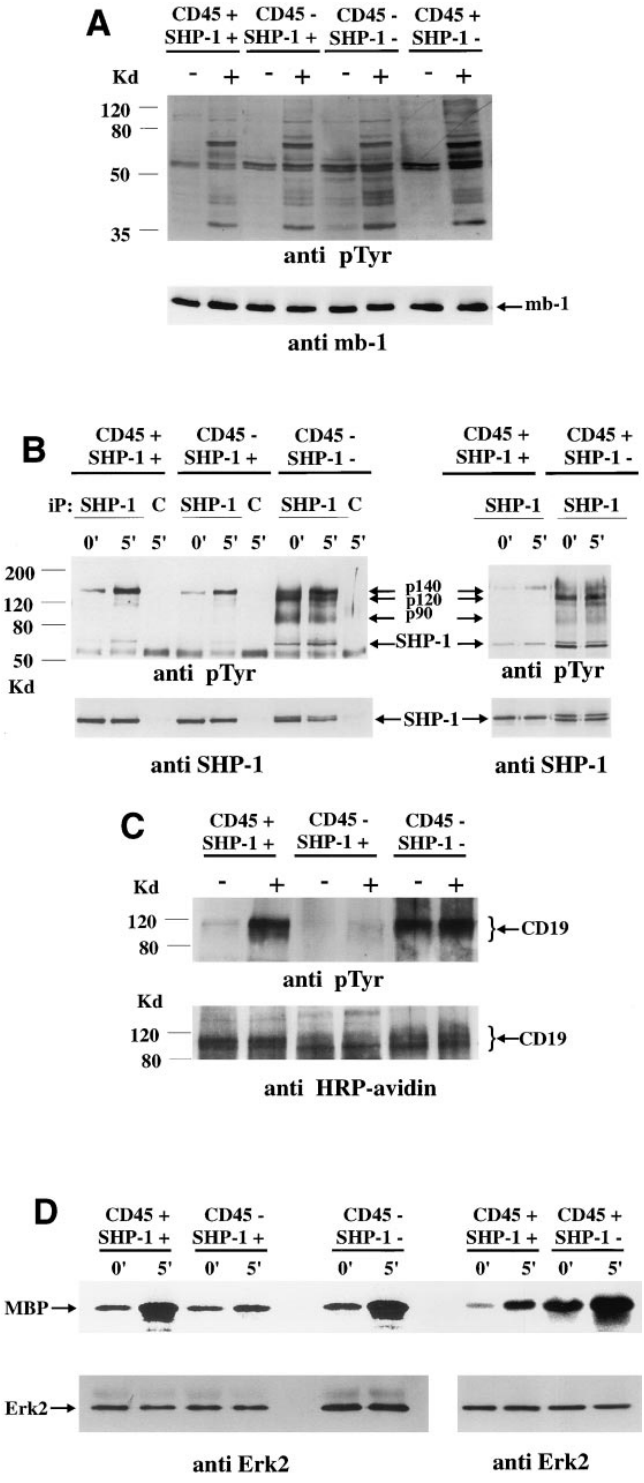

Figure 3. Analysis of SHP-1 phosphoprotein binding and MAP kinase activation in stimulated B cells from PTP-deficient mice. (*A*) Comparison of protein tyrosine phosphorylation in resting and anti-Ig–treated B cells from wild-type ($CD45^+/SHP\text{-}1^+$), $CD45^-CD45^-/SHP\text{-}1^-$, and $CD45^+/SHP\text{-}1^-$ mice. Loading of equivalent amounts of lysate proteins was confirmed by reblotting with anti–mb-1 antibody (*bottom*). (*B*) Antiphosphotyrosine (*anti pTyr*) immunoblots (*top*) showing the tyrosine-phosphorylated species coprecipitated with SHP-1 from resting and anti-Ig–treated B cells from wild-type ($CD45^+/SHP\text{-}1^+$), $CD45^-$, and $CD45^-/SHP\text{-}1^-$ mice (*left*) and from $CD45^+/SHP\text{-}1^-$ mice (*right*). Arrows indicate the positions of SHP-1 and three associated phosphoproteins that appear differentially phosphorylated in the $CD45^-$ compared to $CD45^-/SHP\text{-}1^-$ and $SHP\text{-}1^-$ cells. Mobilities of molecular mass standards are shown on the left. Loading of equivalent amounts of lysate protein was confirmed by reblotting with anti–SHP-1 antibody (*bottom*). Data are representative of three independent experiments on nine mice. (*C*) Antiphosphotyrosine immunoblot (*top*) showing the tyrosine phosphorylation status of CD19 immunoprecipitates derived from biotinylated resting and anti-Ig–treated wild-type, $CD45^-$, and $CD45^-/SHP\text{-}1^-$ cells was carried out as described in Materials and Methods. The position of CD19 is indicated by the arrow on the right. Loading of equivalent amounts of CD19 was confirmed by reblotting with HRP-avidin (*bottom*). (*D*) Representative example showing the levels of MAP kinase activities before and 5 min after BCR ligation in wild-type, $CD45^-$, $CD45^-/SHP\text{-}1^-$, and $CD45^+/SHP\text{-}1^-$ cells (*top*). Analysis of equivalent amounts of Erk-2 was confirmed by anti-Erk2 immunoblotting of equivalent aliquots of each Erk-2 immunoprecipitate (*bottom*).

interpretation is supported by the detection of a phosphoprotein that co-migrates with CD19 in SHP-1 immunoprecipitates, suggesting a capacity for SHP-1 to associate with CD19 or a CD19-bound protein. Thus, augmented phosphorylation of CD19 in the $CD45^-/SHP\text{-}1^-$ cells likely reflects the loss of both SHP-1–driven CD19 dephosphorylation and $CD45^-$ dependent, PTK-mediated CD19 phosphorylation. Since CD19 has been shown to serve as a coreceptor that positively modulates signal transducing capacity of the BCR (32), these data suggest that the counterbalance of CD45 and SHP-1 effects on CD19 recruitment to the BCR signaling cascade represents one biochemical mechanism whereby these PTPs exert their coordinate regulation of BCR signaling capacity. This hypothesis, as well as the identity of the other SHP-1–associated phosphoprotein species appearing hyperphosphorylated in the $CD45^-/SHP\text{-}1^-$ cells, require further investigation. Nonetheless, the suggestion that CD45 and SHP-1 coordinate effects on BCR function are realized at the level of CD19 phosphorylation is consistent with the data indicating a pivotal role for CD19 in modulating the threshold for coupling BCR stimulation to not only proliferation, but also to the development of B-1 cells (24, 33).

In addition to induction of tyrosine phosphorylation, anti-Ig elicited activation of the MAP kinase cascade, a more downstream signaling event that couples BCR-induced Ras activation to nuclear response, was also examined in the PTP-deficient cells (34). As shown in Fig. 3 *D*, analysis of ERK2-immunoprecipitable kinase activities in these cells revealed that the induction of MAP kinase activity after BCR engagement was no different between $CD45^-/SHP\text{-}1^-$ and wild-type cells, but was relatively enhanced in $SHP\text{-}1^-$ cells, and essentially abrogated in $CD45^-$ cells. These results indicate a parallel between MAP kinase activation and the capacities of these PTP-deficient B cells to proliferate in response to BCR ligation. Thus, the molecular interactions which allow SHP-1 and CD45 to cooperatively influence the outcome of BCR engagement appear to occur at or proximal to activation of the MAP kinase cascade.

In summary, our results indicate that SHP-1 deficiency rescues antigen-receptor–elicited proliferation in CD45-deficient mice. The relatively normal splenic B cell phenotype observed in the double mutant mice is most likely a

consequence of the restoration of the selective forces that promote normal B cell maturation in the spleen. It remains to be determined whether the effects observed are directly or indirectly determined by the interactions of the PTPs. It is possible that altered selection at various levels of B cell development results in quite distinct populations of splenic B cells. The signaling defects observed in CD45-deficient B cells suggest that BCR-mediated activation is normally restricted to conditions that allow the engagement of CD45. This restriction appears to be required during the final stages of B cell maturation as transitional cells are recruited into the mature B cell pool, and may serve to restrain the amplitude of the immune response by setting a threshold for B cell activation. A key role for SHP-1 in this regulatory circuit is also apparent as the absence of SHP-1 circumvents the requirement for CD45 in B cell maturation/activation. Thus, the data reported here, which suggest that the balance of SHP-1 and CD45 activities substantially influence the outcome of BCR engagement, identify the biochemical mechanisms whereby these PTPs coordinately modulate BCR activation as being critical to the maintenance of normal B cell responses and the prevention of autoreactivity.

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