

Identification of the Tyrosine Phosphatase PTP1C as a B Cell Antigen Receptor-associated Protein Involved in the Regulation of B Cell Signaling

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Summary

Recent data implicating loss of PTP1C tyrosine phosphatase activity in the genesis of the multiple hemopoietic cell defects found in systemic autoimmune/immunodeficient motheaten (*me*) and viable motheaten (*me^v*) mice suggest that PTP1C plays an important role in modulating intracellular signaling events regulating cell activation and differentiation. To begin elucidating the role for this cytosolic phosphatase in lymphoid cell signal transduction, we have examined early signaling events and mitogenic responses induced by B cell antigen receptor (BCR) ligation in *me* and *me^v* splenic B cells and in CD5⁺ CH12 lymphoma cells, which represent the lymphoid population amplified in motheaten mice. Despite their lack of functional PTP1C, *me* and *me^v* B cells proliferated normally in response to LPS. However, compared with wild-type B cells, cells from the mutant mice were hyperresponsive to normally submitogenic concentrations of F(ab')₂ anti-Ig antibody, and they exhibited reduced susceptibility to the inhibitory effects of FcγIIIRB cross-linking on BCR-induced proliferation. Additional studies of unstimulated CH12 and wild-type splenic B cells revealed the constitutive association of PTP1C with the resting BCR complex, as evidenced by coprecipitation of PTP1C protein and phosphatase activity with BCR components and the depletion of BCR-associated tyrosine phosphatase activity by anti-PTP1C antibodies. These results suggest a role for PTP1C in regulating the tyrosine phosphorylation state of the resting BCR complex components, a hypothesis supported by the observation that PTP1C specifically induces dephosphorylation of a 35-kD BCR-associated protein likely representing Ig-α. In contrast, whereas membrane Ig cross-linking was associated with an increase in the tyrosine phosphorylation of PTP1C and an ~140-kD coprecipitated protein, PTP1C was no longer detected in the BCR complex after receptor engagement, suggesting that PTP1C dissociates from the activated receptor complex. Together these results suggest a critical role for PTP1C in modulating BCR signaling capacity, and they indicate that the PTP1C influence on B cell signaling is likely to be realized in both resting and activated cells.

The recent data linking the murine motheaten phenotype of severe systemic autoimmunity and immunodeficiency to loss-of-function mutations in the gene encoding the PTP1C tyrosine phosphatase (1–3) suggest that this phosphotyrosine

phosphatase (PTP)¹ plays a critical role in regulating hemopoietic cell differentiation and function (1–3). Since PTP1C is expressed in all hemopoietic cell types and contains two src homology 2 (SH2) domains (4–6), its effects on hemopoiesis are likely realized through modulation of a multiplicity of intracellular signaling cascades. This hypothesis is supported by the wide range of hemopoietic cell defects observed in mice homozygous for the motheaten (*me*) or allelic viable motheaten (*me^v*) mutations (7, 8), animals in which PTP1C catalytic activity is negligible consequent to production of no or mutant PTP1C protein, respectively (1).

¹ Abbreviations used in this paper: BCR, B cell antigen receptor; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate; DTSP, dithiobis(succinimidyl propionate); FcγIIIRB, B cell receptor for IgG Fc region; *me*, motheaten; *me^v*, viable motheaten; mIg, membrane Ig; pNPP, *p*-nitrophenyl phosphate; PTK, protein tyrosine kinase; PTP, phosphotyrosine phosphatase; SH2, src homology 2.

PTP1C involvement in a diversity of hemopoietic cell signaling pathways is also suggested by biochemical data showing association of this PTP with the *c-kit*, IL-3, erythropoietin, and CSF-1 receptors (9–13). Although the mechanisms by which PTP1C interacts with and modulates signals evoked by activation of these growth factor receptors have not been delineated, the massive overexpansion of myeloid, monocytic, and erythroid progenitor cells found in *me* and *me^v* mice (14–16) suggests that PTP1C acts to down-regulate proliferation and/or differentiation signals triggered by receptor engagement.

In addition to engendering multiple myelomonocytic cell abnormalities, loss of PTP1C function in *me* and *me^v* mice has a profound effect on B lymphoid ontogeny: the mutant mice show markedly increased numbers of peripheral B-1 or CD5⁺ B lymphocytes, a putatively autoreactive B cell subpopulation that is normally concentrated in the peritoneal and pleural cavities (17–20). The abnormal expansion of the CD5⁺ B cell population in *me* and *me^v* mice and the associated high levels of serum Igs, autoantibodies, and B cell lymphokines in these mice again suggest a regulatory role for PTP1C in relation to induction of B cell activation, and raise the possibility that PTP1C normally functions to down-regulate signaling pathways coupling antigen and/or mitogen stimulation to proliferation. To investigate this possibility, we have examined the response of PTP1C-deficient *me* and *me^v* B cells to B cell antigen receptor (BCR) engagement, and we have evaluated PTP1C interactions with the BCR complex in the murine B cell lymphoma line CH12. The data presented here reveal an increased sensitivity of the motheaten B cells to proliferation signals evoked by membrane Ig (mIg) ligation, and they indicate that PTP1C is physically and functionally associated with the BCR complex in unstimulated B cells. By contrast, BCR engagement appears to disrupt PTP1C association with the antigen-receptor complex while inducing the tyrosine phosphorylation of both PTP1C and an associated 140-kD molecule. Together these results demonstrate an important role for PTP1C in modulating signaling events coupling the BCR to cell activation.

Materials and Methods

Mice. Mice for these studies were obtained by mating C3HeBFeJ *me/+* and *+/+* and C57BL/6J *me^v* and *+/+* breeding pairs derived from breeding stock maintained at the Samuel Lunenfeld Research Institute, Mount Sinai Hospital (Toronto, Canada). All mice used in the study were 3–5 wk old.

Reagents. Antibodies used for these studies include a rat anti-Thy1.2 antibody purified from the hybridoma clone J1j.10 (ATCC T1B184; American Type Culture Collection, Rockville, MD), a rabbit polyclonal anti-PTP1C SH2 domain antibody produced in our lab (1), rabbit polyclonal anti-Syp SH2 domain and antiphosphotyrosine antibodies provided by Dr. A. Pawson (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada) (21), rabbit anti-MB-1 cytoplasmic tail antibody provided by Dr. L. Matsuuchi (Dept. of Zoology, University of British Columbia, Vancouver, Canada) (22), rabbit anti-mouse Ig (μ heavy chain; Serotec Ltd., Toronto, Ontario), affinity-purified F(ab')₂ goat anti-mouse Ig (μ heavy chain Cappel Laboratories,

Durham, NC), and a murine IgM anti-CD5 antibody from the hybridoma line KT25 (Serotec Ltd.). To derive glutathione S-transferase (GST)-PTP1C fusion protein for this work, the full-length PTP1C cDNA (1785 bp) was cloned into pGEX-2T (Pharmacia LKB Biotechnology Inc., Baie d'Urfé, PQ) and transformed into *Escherichia coli* JM101, and the fusion proteins were purified from bacterial lysates after isopropyl β -D-thiogalactopyranoside induction.

Cells and Cell Lines. The CD5⁺ murine B lymphoma line CH12 of 2^a4^b origin (23) (provided by D. A. Kaushik, Depts. of Veterinary Microbiology and Immunology, University of Guelph, Guelph, Ontario) and the Thy1.2⁺ murine lymphoma line EL4 (ATCC T1B39) were cultured in RPMI 1640 (Gibco Laboratories, Grand Island, NY) with 10% FCS (Sterile System Inc., Logan, UT), 50 μ M 2-ME, 2 mM L-glutamine, and penicillin/streptomycin and harvested in the log growth phase. Purified populations of mIg⁺ splenic B lymphocytes were obtained from *me*, *me^v*, and wild-type congenic (*+/+*) mice by subjecting splenic cell suspensions to erythrocyte lysis in 0.8% ammonium chloride, followed by treatment with rat anti-Thy1.2 antibody for 30 min on ice and subsequent incubation with a 1:15 dilution of rabbit complement (Serotec Ltd.) in RPMI 1640 for 45 min at 37°C. The T cell-depleted splenocytes were then washed in HEPES-buffered saline and layered on a 75/60/50% Percoll gradient (Pharmacia), and high density resting B lymphocytes were recovered from the 75/60% Percoll interface. The resulting cells were >90% mIg⁺ as determined by FACS[®] (Becton Dickinson, Mountain View, CA) analysis.

Proliferation Assays. Splenic B cells were resuspended in IMDM (Gibco Laboratories) containing 10% FCS, 50 μ M 2-ME, 2 mM L-glutamine, antibiotics, and 100 U/ml IL-4 (Sigma Chemical Co., St. Louis, MO), and they were cultured in quadruplicate at a final concentration of 5×10^4 cells per 100 μ l in flat-bottomed microtiter plates in the presence or absence of varying concentrations of F(ab')₂ goat anti-Ig antibody, 20 μ g/ml intact rabbit anti-Ig antibody, or 20 μ g/ml LPS (Sigma Chemical Co.). Cultures were continued for 48 h and pulsed with [³H]thymidine (1 μ Ci per well) 6 h before terminating incubation. [³H]Thymidine incorporation was measured using an automated β liquid scintillation counter.

Cell Stimulation and Lysis. CH12 cells (5×10^7) or purified splenic B cells (2×10^7) were resuspended in 5 ml of culture medium and stimulated with 25 μ g/ml F(ab')₂ anti-Ig antibody for varying periods of time (CH12 cells) or with 50 μ g/ml anti-Ig antibody for 10 min (splenic B cells). Cells were stimulated at 37°C, washed in 9 ml of cold PBS with 1 mM sodium orthovanadate, and resuspended in 1 ml of lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8, 2 mM EDTA, 50 mM NaF, 1 mM PMSF, 1 mM sodium orthovanadate) containing either 5 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS) (Sigma Chemical Co.) or 1% NP-40 as detergents. Nuclei and unlysed cells were removed by centrifugation at 4°C for 10 min at 14,000 g. For cross-linking studies, 5×10^7 CH12 cells were incubated for 30 min with 20 μ g/ml dithiobis(succinimidyl propionate) (DTSP; Sigma Chemical Co.) in PBS at 4°C and washed twice in cold PBS plus NaN₃ before lysis.

Immunoprecipitation and Immunoblotting. Lysates were precleared before immunoprecipitation by incubating 1 mg of cell lysate protein in a volume of 1 ml with 40 μ l of protein A-Sepharose beads (Pharmacia) for 1 h at 4°C and then with 40 μ l of beads and 5 μ l of rabbit preimmune serum for an additional 1 h. Lysates were then incubated for 2 h at 4°C with 5 μ l of anti-PTP1C antibody, 5 μ l of intact anti-mouse Ig antibody, or 5 μ l of rabbit preimmune serum and 10 μ l of protein A-Sepharose. Immunocomplexes were collected by centrifugation (5 min at 10,000 g) and washed four

times in lysis buffer, and the pellets were resuspended in SDS sample buffer. Samples were boiled for 5 min and electrophoresed through 8% SDS-polyacrylamide gels, and the separated proteins were then transferred to nitrocellulose (Bio Rad Laboratories, Mississauga, Ontario). Specific proteins were detected by immunoblotting with anti-PTP1C, anti-phosphotyrosine, or anti-MB-1 antibodies, followed by incubation with ^{125}I -protein A (Dupont-NEN, Boston, MA) in 10 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% Tween 20 and exposure to XAR film (Eastman Kodak Co., Rochester, NY) at -70°C .

Assays of Phosphatase Activity. For analysis of BCR-associated phosphatase activity, anti-Ig immunoprecipitates were prepared as previously described from CHAPS lysates of 10^8 unstimulated EL4 cells (control) or from 2.5, 5, or 10×10^7 CH12 cells incubated for 15 min in medium alone, medium containing 50 μl mouse IgM (1:20 dilution of KT25 hybridoma supernatant), or medium containing 25 $\mu\text{g}/\text{ml}$ $\text{F}(\text{ab}')_2$ anti-Ig antibody. Phosphatase assays were also performed on anti-Ig immunoprecipitates prepared from CHAPS lysates of 5×10^7 CH12 cells immunodepleted of PTP1C or Syp by overnight incubation with an excess (50 μl) of anti-PTP1C or anti-Syp antibodies, respectively, followed by addition of 100 μl protein A-Sepharose. Immunoprecipitates were washed three times in lysate buffer and then incubated at 37°C for 12 h in 200 μl phosphatase buffer (62 mM Hepes, pH 5.0, 6.25 mM EDTA, 12.5 mM DTT) containing 4 mM *p*-nitrophenyl phosphate (pNPP; Sigma Chemical Co.). Reactions were terminated by addition of 0.8 ml of 200 mM NaOH, and absorbance was measured at 410 nm by spectrophotometry. For some experiments, reactions were performed in 40 μl phosphatase buffer and terminated by addition of 0.16 ml of 200 mM NaOH; absorbance was measured at 410 nm by ELISA.

To assay PTP1C-induced dephosphorylation of BCR-associated proteins, 5×10^7 unstimulated CH12 cells were suspended in lysis buffer with 1% digitonin, and the lysates were subjected to anti-Ig immunoprecipitation as previously described. Immunoprecipitates were washed three times in lysis buffer and once in kinase buffer (20 mM Hepes, NaOH, pH 7.4, 10 mM MgCl_2), resuspended in 25 μl of kinase buffer, and then incubated for 30 min at room temperature with 10 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ ($>3,000$ Ci/mol; Amersham Corp., Arlington Heights, IL). The reaction was terminated by addition of 1 ml of cold phosphatase buffer, and the precipitates were recovered by centrifugation and resuspended in 25 μl phosphatase buffer containing either 5 μg of the full-length GST-PTP1C fusion protein bound to Sepharose or Sepharose alone (control). After overnight incubation at 37°C , the reaction was stopped by addition of 25 μl of 2 \times electrophoresis sample buffer, and the labeled proteins were resolved by SDS-PAGE (8% gel) and detected by autoradiography.

Results and Discussion

To investigate the potential relevance of PTP1C activity to BCR-evoked activation, the effects of BCR cross-linking induced by anti-Ig antibody on lymphocyte proliferation were compared in *me* and *me^v* PTP1C-deficient and wild-type congenic mice. As measured by ^3H thymidine incorporation, the maximal proliferative responses to $\text{F}(\text{ab}')_2$ anti-Ig treatment were similar in magnitude in motheaten and control cells (Fig. 1 A), and they were detected at the same time point after stimulation (48 h) in the wild-type and mutant cell cultures. However, as indicated by the dose-response profiles shown in Fig. 1 A, peak proliferative responses oc-

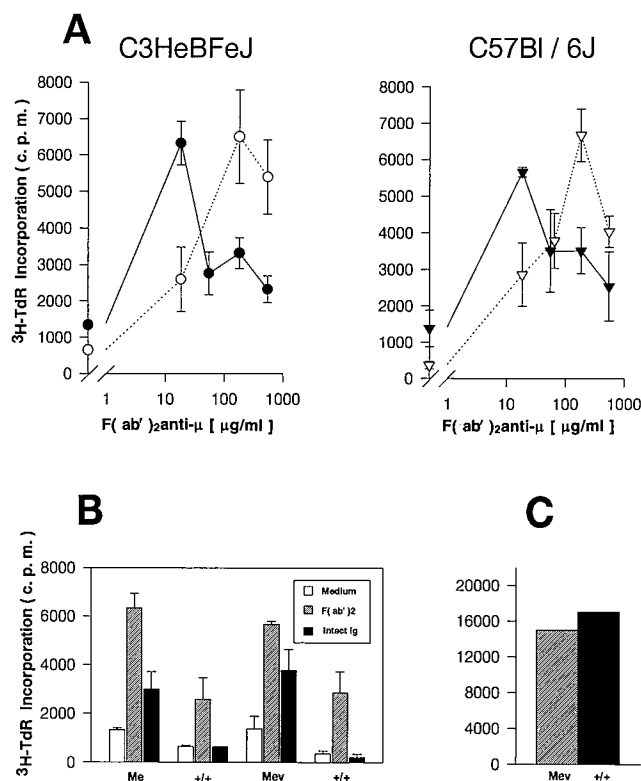


Figure 1. Effect of anti-Ig and LPS on proliferation of *me*, *me^v*, and normal splenic B cells. (A) Splenic B cells (5×10^4 per well) purified from C3HeBFeJ wild-type (O), *me* homozygous (●), and C57Bl/6J wild-type (∇) or *me^v* heterozygous (\blacktriangledown) mice were cultured for 48 h with the indicated amounts of $\text{F}(\text{ab}')_2$ goat anti-Ig, and proliferation was evaluated after a 6-h pulse with ^3H thymidine. (B) Splenic B cells purified from *me* and *me^v* homozygous, and congenic wild-type (+/+) mice were cultured for 48 h in the presence of 15 $\mu\text{g}/\text{ml}$ $\text{F}(\text{ab}')_2$ goat anti-Ig, 20 $\mu\text{g}/\text{ml}$ intact rabbit anti-Ig, or medium alone, and they were pulsed with ^3H thymidine for the last 6 h of culture. (C) Splenic B cells purified from *me^v* homozygous or C57Bl/6J (+/+) mice were cultured for 48 h in the presence of 20 $\mu\text{g}/\text{ml}$ LPS and pulsed with ^3H thymidine for the last 6 h of culture. Data are expressed as cpm, and values represent means (\pm SEM) of quadruplicate cultures.

curring in the mutant B cells at antibody concentrations 5–10 times lower than those inducing peak responses in normal cells (15 versus 150 $\mu\text{g}/\text{ml}$, respectively). Similarly, when stimulated with smaller amounts of anti-Ig (i.e., at concentrations <15 $\mu\text{g}/\text{ml}$), *me* and *me^v* B cell proliferative responses were consistently greater in magnitude than those observed in control mice. In contrast, at the anti-Ig dose range which stimulated maximal responses in normal B cells, the *me* and *me^v* B cell proliferative responses were relatively reduced. The increased responsiveness of the motheaten B cells relative to control cells was not a generalized phenomenon since proliferative responses to LPS were comparable in the mutant and wild-type splenic cells (Fig. 1 C). Moreover, the apparent shift in the anti-Ig dose-response profile observed in *me* and *me^v* B cells is unlikely to reflect differential expression of mIg on motheaten versus control cells, as results of immunofluorescence analyses by our group (unpublished observations) and others (16, 24) have indicated levels of mIgM

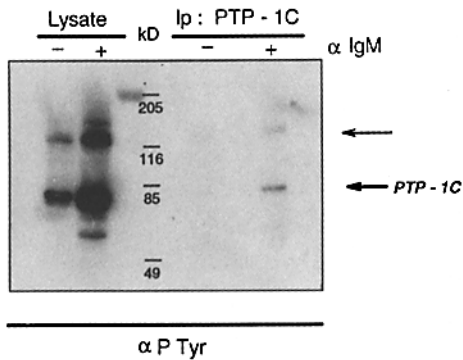


Figure 2. Anti-phosphotyrosine (αP Tyr) immunoblots showing the effect of mIg cross-linking on protein tyrosine phosphorylation in splenic B cells. Cell lysates were prepared from 2×10^7 unstimulated (-) or F(ab')₂ anti-Ig (50 μ g/ml)-stimulated (+) normal splenic B cells. Cell lysates alone (Lysate) or lysates immunoprecipitated with anti-PTP1C antibody (Ip:PTP1C) were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with anti-phosphotyrosine antibody. The positions of PTP1C and a second tyrosine-phosphorylated protein are indicated by arrows, and the mobilities of molecular mass standards are shown.

to be, if anything, slightly lower on motheaten relative to normal B cells. Similarly, the results cannot be ascribed to the presence of a largely CD5⁺ peripheral B cell population in motheaten mice compared with the predominance of CD5⁻ B cells in normal mice, as we (data not shown) and others (25) have found that in normal mice, the capacity of anti-Ig to elicit early activation events is substantially reduced in CD5⁺ compared with CD5⁻ B cells. Together these observations suggest that the reduced threshold for anti-Ig-induced proliferation observed in motheaten B cells represents an abnormality specific to BCR-mediated signal transduction and by extension imply a role for PTP1C in modulating signaling events coupling BCR engagement to proliferation.

In contrast to the findings presented here, data from several earlier studies suggested a severe reduction in *me* and *me*^v B cell mitogenic responses (24, 26). These latter studies, however, involved analyses of whole splenic cell populations and the consequent exposure of the motheaten B cells to other hematopoietic cells affected by the mutation, most notably the expanded and putatively immunosuppressive myelomonocytic population (27, 28). By contrast, more recent data showing that phorbol ester responsiveness is only slightly reduced in purified *me*^v splenic B cells compared with CD5⁺ B cells from normal mice (29) indicate that motheaten B cells maintain the ability to proliferate in response to at least some mitogenic stimuli, and suggest that the defect responsible for increased anti-Ig responses in these cells acts at a very proximal level, modifying the signaling events associated with BCR engagement before activation of protein kinase C. This postulate is supported by the data shown in Fig. 1 B, which indicate that motheaten B cells are also distinguished from normal B cells by their proliferative responses to treatment with intact anti-Ig, which in contrast to F(ab')₂ anti-Ig, co-cross-links mIg with the B cell Fc receptor, Fc γ R1IB. Previous data concerning Fc γ R1IB involvement in B cell signaling have revealed that Fc γ R1IB-mIg cross-linking suppresses

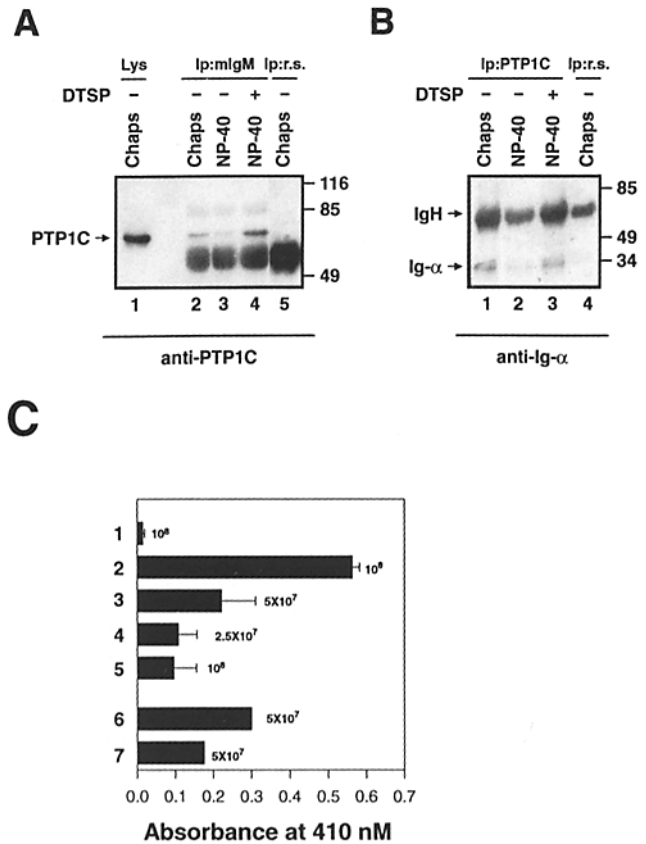


Figure 3. Association of PTP1C protein and phosphatase activity with the BCR. (A and B) Unstimulated CH12 cells (5×10^7) were lysed in 5 mM CHAPS, 1% NP-40, or 1% NP-40 after pretreatment with 20 μ g/ml DTSP. Lysates (1 mg protein) were immunoprecipitated with anti-Ig (A), anti-PTP1C (B), or rabbit preimmune serum (r.s.), and lysate (Lys) or immunoprecipitated lysate proteins were analyzed by SDS-PAGE and immunoblotting with anti-PTP1C (A) or anti-Ig α (B) antibodies. The positions of PTP1C, Ig heavy chain (IgH), and Ig α are indicated by arrows, and the mobilities of size markers (in kilodaltons) are shown on the right. (Note: lanes have been reorganized solely for clarity and to remove extraneous data and are from a single autoradiogram.) (C) Cell lysates were prepared from 10^8 EL4 cells (lane 1), from 10^8 (lanes 2 and 5), 5×10^7 (lane 3), or 2.5×10^7 (lane 4) unstimulated CH12 cells, and from CH12 cells pretreated with an excess of anti-Syp (lane 6) or anti-PTP1C (lane 7) antibodies. Lysates were immunoprecipitated with anti-Ig antibody or anti-Ig antibody plus an excess of mouse IgM (lane 5), and the precipitated proteins were incubated for 12 h with 4 mM pNPP at 37°C. After addition of NaOH, absorbance was measured at 410 nm using an ELISA plate reader. The results are representative of five (lanes 1-5) or two (lanes 6 and 7) independent experiments. Bars indicate standard deviations for experiments performed in duplicate.

early activation signals generated by BCR engagement, an effect apparently mediated through interactions between Fc γ R1IB and the Ig- α and - β components of the BCR complex (30). As shown in Fig. 1 B, this phenomenon was also apparent in the wild-type cells studied here, which showed negligible proliferation after incubation with intact anti-Ig. By contrast, whereas the response of *me* and *me*^v cells to intact anti-Ig was also less than that induced by comparable amounts of F(ab')₂ anti-Ig, the inhibitory effect of cross-linking Fc γ R1IB and mIg was much less striking in these cells

than in the wild-type B cells. These results provide further evidence for a role of PTP1C in modulating the molecular events relevant to propagation of the BCR-evoked proliferation signal.

To address further the involvement of PTP1C in BCR-driven mitogenesis, the possibility that this phosphatase, like many other cytoplasmic proteins implicated in mIg-mediated signaling (31, 32), undergoes tyrosine phosphorylation after receptor engagement was examined in anti-Ig-treated splenic B cells from normal mice. As determined by antiphosphotyrosine immunoblotting of total cell lysates, anti-Ig treatment induced marked increases in the tyrosine phosphorylation of a number of intracellular proteins (Fig. 2). However, although the amounts of PTP1C immunoprecipitated from resting and stimulated cells were comparable (data not shown), tyrosine phosphorylation of this 67-kD PTP was detected only in immunoprecipitates derived from anti-Ig-treated cells. Similarly, an ~140-kD protein species coprecipitated with PTP1C was also tyrosine phosphorylated only after mIg ligation. Although its identity has not been determined, this latter phosphoprotein may represent CD22, a BCR-associated adhesion molecule that has been reported to associate with PTP1C after BCR engagement (33). In any case, the induction of PTP1C tyrosine phosphorylation after mIg ligation provides additional evidence for PTP1C involvement in the intracellular signaling events elicited by BCR engagement.

These initial observations implicating PTP1C in BCR-mediated signal transduction raised the possibility that PTP1C might physically associate with proteins making up the BCR complex. To determine whether PTP1C interacts with the BCR constituents, i.e., mIg and the associated Ig- α (MB-1) and Ig- β (B29) heterodimer, the presence of PTP1C was evaluated in anti-Ig immunoprecipitates prepared from CH12 cells, a murine B lymphoma phenotypically similar (CD5⁺, IgM^{hi}, IgD^{lo}) to *me* and *me^v* B cells (23). As shown in Fig. 3 A, PTP1C was detectable in anti-Ig immunoprecipitates from CHAPS lysates of unstimulated CH12 cells. As is consistent with previous data showing disruption of the BCR complex components by harsh detergents (34), less PTP1C protein was detected in anti-Ig immunoprecipitates from CH12 lysates prepared with NP-40, whereas coprecipitation of PTP1C with mIg was increased when NP-40 lysates were prepared from cells pretreated with DTSP, a chemical cross-linker previously shown to stabilize the mIg/Ig α / β complex (35). PTP1C interaction with mIg is not restricted to CH12 cells since association of these proteins was also observed in unstimulated fresh splenic B cells and in the CD5⁻ WEHI-231 lymphoma cells (data not shown). Moreover, in the reciprocal experiment, mIg was also detected in anti-PTP1C immunoprecipitates from the CH12 cells (data not shown). Similar results were also observed with respect to Ig α , which coprecipitated more efficiently with PTP1C from CHAPS and NP-40/DTSP than from NP-40 lysates of CH12 cells (Fig. 3 B). These observations suggest that PTP1C binds to the unstimulated BCR, either directly, by association with the Ig- α / β heterodimer, or indirectly, by interaction with other molecules associated with the resting receptor complex, such as the protein tyrosine kinases (PTK), lyn, fyn, and blk

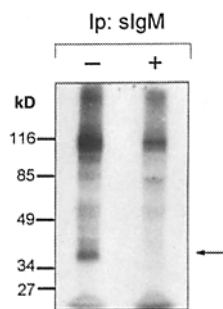


Figure 4. In vitro tyrosine dephosphorylation of BCR-associated proteins by GST-PTP1C fusion protein. Lysates from 5×10^7 unstimulated CH12 cells were immunoprecipitated with anti-Ig antibody, and the immunoprecipitated proteins were tyrosine phosphorylated in vitro (see Materials and Methods) and incubated with 5 μ g Sepharose-bound GST-PTP1C (+) or Sepharose alone (-). Immunoprecipitated proteins were subjected to SDS-PAGE, and phosphorylation was visualized by autoradiography. The arrow indicates the position of an ~35-kD species apparently selectively tyrosine dephosphorylated after incubation with GST-PTP1C.

(36–38), all of which have been reported to bind to Ig α in unstimulated B cells (39). Although both the Ig α / β subunits contain several tyrosine phosphorylation sites that represent potential targets for SH2 domain-mediated protein binding, the association of PTP1C with the receptor complex occurs in resting cells, in which these components are phosphorylated at only low levels (21). This observation, in conjunction with the relative instability of the PTP1C mIg/Ig α association in NP-40, suggests that PTP1C binding to the resting BCR is not mediated through the interaction of PTP1C SH2 domains with phosphotyrosine.

To determine whether the PTP1C protein associated with the unstimulated BCR complex is enzymatically active, anti-Ig immunoprecipitates from CH12 cells were evaluated for the presence of tyrosine phosphatase activity. As shown in Fig. 3 C, these immunoprecipitates displayed a cell dose-dependent capacity to dephosphorylate pNPP substrate, a finding that suggests the coprecipitation of a catalytically active phosphatase. To assess the extent to which PTP1C accounts for anti-Ig immunoprecipitable phosphatase activity, this experiment was also performed on lysates from CH12 cells immunodepleted of either PTP1C or the structurally similar tyrosine phosphatase Syp (21). Results from this analysis revealed BCR-associated phosphatase activity to be unaffected by Syp immunodepletion, but markedly reduced in PTP1C-immunodepleted samples (Fig. 3 C). Although these data do not preclude the contribution of PTPs other than PTP1C to the tyrosine phosphatase activity detected in BCR immunoprecipitates, the findings suggest that PTP1C accounts for a significant proportion of this activity and as such may act to suppress or minimize tyrosine phosphorylation of the Ig α / β subunits or other BCR-associated proteins in resting cells. To pursue this issue, anti-Ig immunoprecipitable proteins from resting CH12 cells were tyrosine phosphorylated in vitro and incubated with GST-PTP1C fusion protein. As shown in Fig. 4, PTP1C specifically dephosphorylated at least one mIg-associated protein of ~35 kD, which may represent Ig α . Although the physiologic relevance of this observation is unclear, the data indicate that Ig α may represent a substrate for PTP1C activity in resting B cells and that PTP1C may therefore serve to counteract effects of PTK activity on the receptor components, thereby maintaining the BCR in a dephosphorylated state.

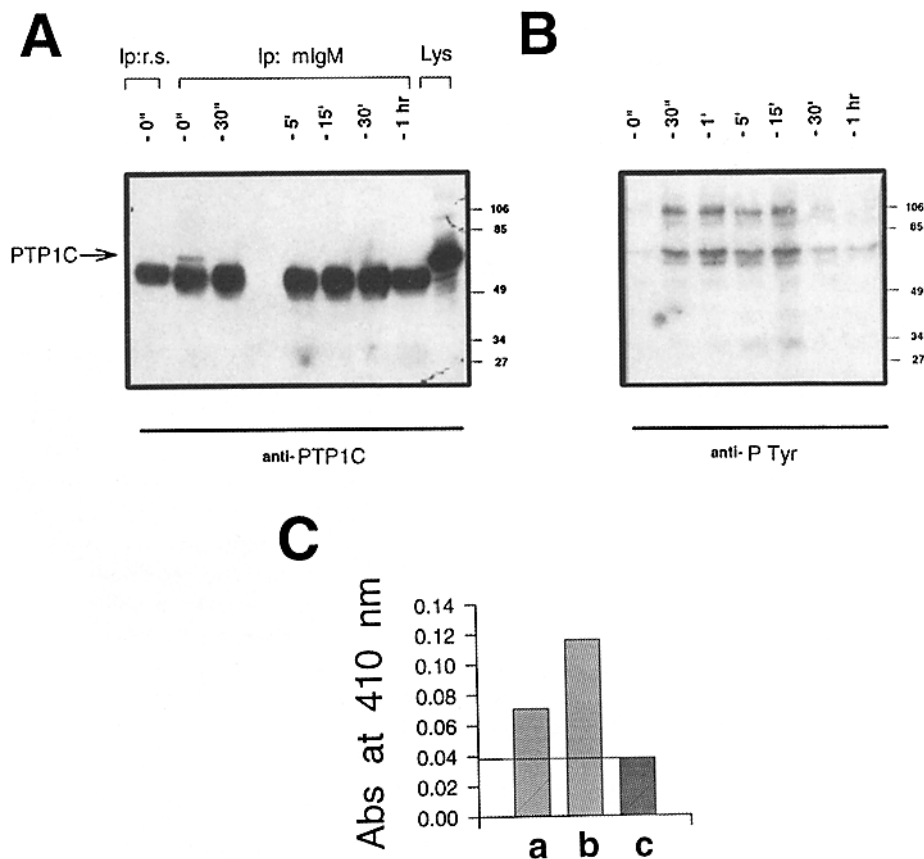


Figure 5. Effect of cross-linking mIgM on PTP1C association with the BCR. (A) At the indicated times after addition of 25 $\mu\text{g/ml}$ anti-Ig antibody, lysates prepared from 5×10^7 CH12 cells were immunoprecipitated with anti-Ig antibody or rabbit preimmune serum (*r.s.*), and the lysate (*Lys*) or immunoprecipitated lysate proteins were resolved by SDS-PAGE and immunoblotting with anti-PTP1C antibody. The migration of PTP1C just above the Ig heavy chain is shown by the arrow on the left. (B) 20- μl aliquots of the same lysates used in experiment A were analyzed by SDS-PAGE and immunoblotting with anti-phosphotyrosine antibody. (C) Lysates were prepared from 5×10^7 CH12 cells incubated for 15 min at 37°C with (lane *a*) or without (lane *b*) 25 $\mu\text{g/ml}$ anti-Ig antibody, or with rabbit preimmune serum (lane *c*). Lysates were subjected to anti-Ig immunoprecipitation and the precipitated proteins were incubated for 12 h at 37°C with 4 mM pNPP. After addition of NaOH, absorbance was measured spectrophotometrically at 410 nm. The data shown are representative of three independent experiments.

To investigate further the influence of PTP1C on BCR signaling capacity, we next examined the effect of anti-Ig stimulation on association between PTP1C and the BCR complex. As indicated by anti-PTP1C immunoblot analysis of anti-Ig-immunoprecipitates from anti-Ig treated CH12 cells, BCR engagement induced a rapid dissociation of the PTP1C-BCR complex: PTP1C was nondetectable in the immunoprecipitates by 30 s after cell stimulation (Fig. 5 A). As is consistent with dissociation of PTP1C from the activated receptor complex, anti-Ig-immunoprecipitable phosphatase activity was also appreciably diminished at 15 min after receptor engagement (Fig. 5 C). These results cannot be ascribed to impaired solubilization of the activated receptor complex or failure to induce receptor activation, as the amounts of Ig α immunoprecipitated under the same conditions were essentially the same in resting and activated B cells (data not shown), and anti-Ig induction of intracellular protein tyrosine phosphorylation was observed in aliquots of the same cell lysates used to examine PTP1C-BCR complex association (Fig. 5 B). Interestingly, whereas levels of tyrosine phosphorylation appeared to peak at about 15 min after receptor stimulation and decline thereafter, PTP1C association with the BCR remained undetectable 15 min after stimulation, and reassociation was first detected only at 30 min after activation. Whether this finding indicates a negative influence of tyrosine phosphorylation on the interaction between PTP1C and the receptor components remains to be determined. Regardless of the mechanism, the rapid disruption of the

PTP1C interaction with the BCR complex after receptor activation, as revealed by the data shown here, provides a means to remove the putatively negative influence of PTP1C on BCR tyrosine phosphorylation and signal amplification. Delayed reassociation of PTP1C with the receptor complex may in turn facilitate termination of the activation signal, thereby contributing to the transient nature of early activation events in B cells.

The observed association between lack of PTP1C function and increased sensitivity of the BCR to ligand-binding proliferation signals, together with the demonstrated capacity of PTP1C to physically bind and to potentially dephosphorylate components of the antigen-receptor complex in unstimulated B cells, identifies PTP1C as an important effector in the modulation of BCR signaling function. The demonstration that this phosphatase associates with the resting receptor complex implies that PTP1C effects on BCR signal propagation are realized, at least in part, at the level of the receptor complex and may reflect the capacity of this phosphatase to counteract PTK-induced tyrosine phosphorylation of the receptor and/or associated signaling molecules. This contention is consistent with data showing that PTKs associate with the resting receptor complex (36-38) and may therefore tyrosine phosphorylate receptor subunits, albeit at low levels, thereby potentially rendering the receptor refractory to future stimulation. Thus, in contrast to the BCR-associated PTKs that promote receptor signaling largely by virtue of their activation after BCR stimulation, the influence of

PTP1C, at least on BCR components per se, may be principally an inhibitory effect realized before receptor engagement. A similar role has also been ascribed to the receptor tyrosine phosphatase CD45 (40). However, since direct physical association of CD45 with the resting BCR complex has not been reported and PTP1C immunodepletion from anti-Ig immunoprecipitates essentially abrogates BCR-associated phosphatase activity, it appears likely that maintenance of a tyrosine-dephosphorylated receptor complex in resting cells is achieved largely through PTP1C activity.

Although receptor engagement appears to result in physical dissociation of PTP1C from the BCR complex, the current as well as other reported data (30) suggest that mIg ligation is followed by PTP1C binding to several other proteins implicated in BCR-mediated signaling, specifically the transmembrane molecules CD22 and Fc γ RIIB, which become tyrosine phosphorylated after cross-linking of mIg or mIg-Fc γ RIIB, respectively (30, 33). In contrast to PTP1C association with the resting BCR, its binding to these latter proteins appears to be mediated through its SH2 domains (reference 33 and unpublished data). By analogy, whereas coupling of PTKs such as fyn and lyn to the stimulated BCR Ig α / β heterodimer is mediated primarily through PTK SH2 domain binding to Ig α / β phosphotyrosine (39), the associa-

tion of these molecules in resting cells does not appear to involve SH2-phosphotyrosine interactions (39). In a similar fashion, PTP1C displaced from the BCR complex after receptor engagement may be recruited via its SH2 domains to associations with other transmembrane or more downstream cytoplasmic signaling effector molecules and thereby influence BCR signaling capacity via several distinct molecular interactions. Although the molecular basis for such interactions requires further definition, it appears likely that PTP1C exerts a predominantly negative influence on signal propagation through the BCR. This putative inhibitory influence of PTP1C on receptor-driven B cell mitogenesis is consistent with the phenotype of B cell hyperactivity observed in motheaten mice, as well as the tendency of *me^v* heterozygous animals to develop B cell lymphomas over time, possibly consequent to functional inactivation of the normal PTP1C allele (16). Therefore, though further studies are required to elucidate fully the effects of PTP1C on antigen receptor-directed signaling in conventional as well as CD5⁺ B-1 cells and the mechanisms by which abrogated PTP1C function translates to overexpansion of the normally minor B-1 population, the data presented here provide compelling evidence of a critical role for PTP1C in the regulation of B cell signaling.

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