

The Tyrosine Phosphatase PTP1C Associates with Vav, Grb2, and mSos1 in Hematopoietic Cells*

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The association of the murine motheaten phenotype of severe hemopoietic dysregulation with loss of PTP1C tyrosine phosphatase activity indicates a critical role for this SH2 domain-containing phosphotyrosine phosphatase in the regulation of hemopoietic cell growth and differentiation. To explore the molecular basis for PTP1C effects on hematopoiesis, we have investigated the possibility that this enzyme interacts with the product of the Vav proto-oncogene, a putative guanine nucleotide exchange factor expressed exclusively in hemopoietic cells. Our data indicate that PTP1C physically associates with Vav in murine spleen cells and in EL4 T lymphoma and P815 mastocytoma cells, and that this interaction is increased following mitogenic stimulation and the induction of both PTP1C and Vav tyrosine phosphorylation. The results also reveal tyrosine phosphatase activity to be present in Vav immunoprecipitates from stimulated splenic and P815 cells and suggest that a major portion of total cellular PTP1C catalytic activity is associated with Vav. As Vav-associated tyrosine phosphatase activity was not detected in PTP1C-deficient motheaten splenic cells, it appears that PTP1C accounts for most, if not all, Vav-coprecipitable tyrosine phosphatase activity in normal cells. The data also demonstrate the capacity of the Vav SH2 domain alone to bind to PTP1C in activated P815 cells, but suggest a role for the two Vav SH3 domains in enhancing this interaction. In addition, the results reveal PTP1C association with two other molecules implicated in Ras activation, the Grb2 adaptor protein and mSos1, a GTP/GDP exchanger for Ras. PTP1C therefore has the capacity to bind and potentially modulate various signaling effectors involved in activation of Ras or Ras-related proteins, and, accordingly, regulation of Ras activation represents a possible mechanism whereby PTP1C influences hemopoietic cellular responses.

Among the phosphotyrosine phosphatases (PTP)¹ identified to date, the cytosolic enzyme PTP1C is distinguished by its predominant expression in hemopoietic cells and the presence of two N-terminal located Src homology 2 (SH2) domains, a motif found in only two other PTPs, Syp (PTP1D/SHPTP2) and the *Drosophila csw* protein (1–6). These properties, together with the recent data linking PTP1C gene mutations to the profound hemopoietic dysregulation manifested by motheaten (*me*) and viable motheaten (*me*^v) mice (7–9), reveal a critical role for PTP1C in modulating hemopoietic cell differentiation and growth. As this PTP has been shown to associate with the activated c-kit, erythropoietin, and IL-3 receptors (10–12) and, more recently, with the B cell antigen receptor complex and the CD22 and FcγRIIB1 receptors on lymphocytes (13–15), PTP1C appears to subserve its regulatory role, at least in part, by modulating the signaling capacities of membrane growth factor/antigen/cytokine receptors. As is consistent with the marked overexpansion of multiple hemopoietic cell types observed in PTP1C-deficient motheaten mice, the data concerning PTP1C effects on the B cell antigen (13–16) and IL-3 (11) receptors suggest that this phosphatase down-regulates signaling cascades elicited by receptor engagement, presumably by dephosphorylating and deactivating receptor components or receptor-associated cytosolic protein tyrosine kinases. In conjunction with the increased susceptibility of *me*^v heterozygous mice to development of lymphoid malignancies (8, 17) and the implicit possibility that PTP1C has tumor suppressor activity, these data suggest that the major influence of PTP1C activity on hematopoiesis may be realized through the suppression of signaling pathways that normally promote cell activation.

In contrast to PTP1C association with specific cell surface receptors, its interactions with downstream cytoplasmic signaling effectors have not been defined. In this regard, one molecule of potential interest is the 95-kDa product of the Vav proto-oncogene, another SH2 domain-containing protein which, like PTP1C, has been identified in all hemopoietic lineages and implicated by several lines of evidence in the control of hemopoietic cell growth and differentiation (18–20). Inhibition of Vav expression, for example, interferes with development of hemopoietic cells from embryonic stem cells (21), and, as is consistent with its participation in a broad range of hemopoietic cell signaling pathways, Vav has been shown to become tyrosine-phosphorylated following cross-linking of antigen receptors on lymphocytes (22–24), Fcγ and ε receptors on monocytes and mast cells, respectively (24, 25), and *c-kit* receptors on multiple hemopoietic lineages (26, 27). Vav contains a number of structural motifs found in many signaling effectors, including an SH2, a pleckstrin homology, and two SH3 do-

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¹ The abbreviations used are: PTP, phosphotyrosine phosphatase(s); GEF, guanine nucleotide exchange factors; ConA, concanavalin A; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis.

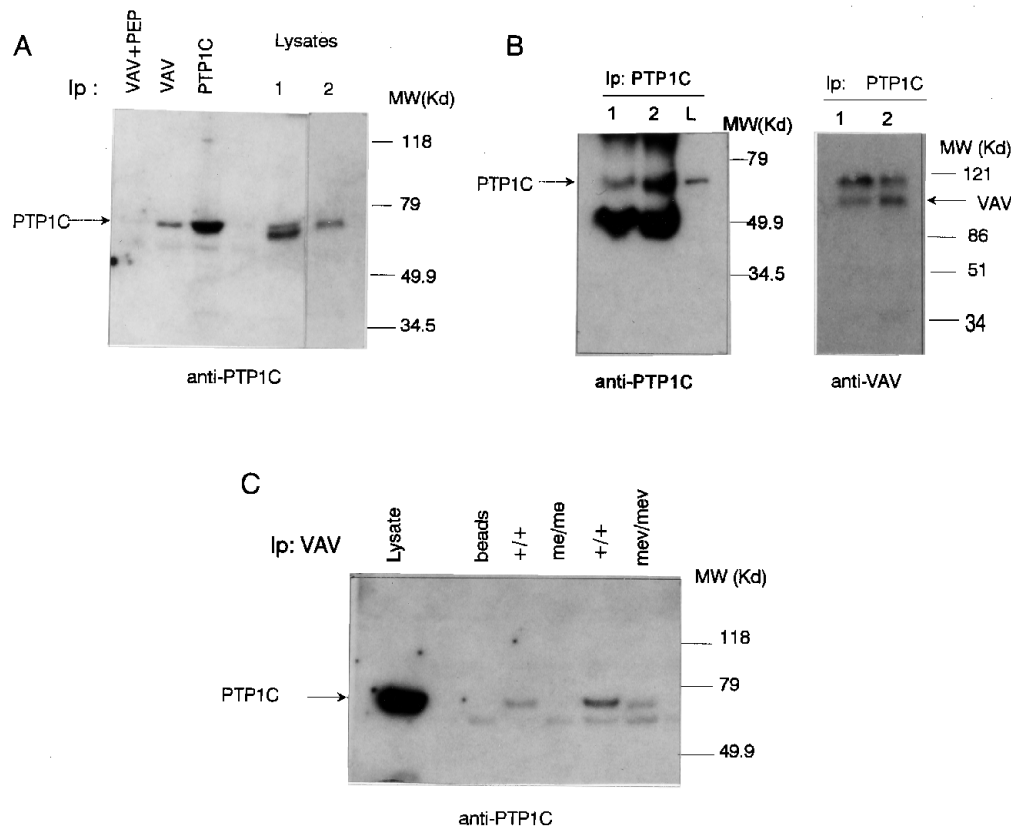


FIG. 1. Association of PTP1C with Vav in hemopoietic cells. A, cell lysates were prepared from 10^8 unstimulated P815 cells and $1500 \mu\text{g}$ of lysate protein, then immunoprecipitated with anti-Vav antibody plus Vav peptide (VAV + PEP), anti-Vav antibody alone, or anti-PTP1C antibody. Lysates were also prepared from 10^8 C57B1 $+/+$ splenic cells (lane 1) and 10^8 EL4 lymphoma cells (lane 2), and the lysate proteins ($\sim 1000 \mu\text{g}$) were resolved on SDS-PAGE and blotted with anti-PTP1C antibody and ^{125}I -protein A. B, cell lysates were prepared from 10^8 unstimulated P815 cells, and $800 \mu\text{g}$ (lane 1) or $1500 \mu\text{g}$ (lane 2) of lysate protein were immunoprecipitated using anti-PTP1C antibody (left panel) or anti-Vav (right panel) antibodies. Lysates ($500 \mu\text{g}$) prepared from P815 cells (L) were also resolved on SDS-PAGE and immunoblotted with anti-PTP1C antibody and ^{125}I -protein A. C, cell lysates were prepared from separated splenic cells of C3HeBFeJ $+/+$ and *me/me* and C57B1/6J $+/+$ and *me^v/me^v* mice, and the lysate proteins ($1500 \mu\text{g}$) were immunoprecipitated with anti-Vav antibody, resolved on SDS-PAGE, and blotted with anti-PTP1C antibody. Aliquots ($1000 \mu\text{g}$) of lysate alone (far left) and lysate plus Sepharose (beads) were also blotted with anti-PTP1C antibody. In all three panels, the positions of molecular mass markers are shown on the right; positions of PTP1C and Vav are indicated by arrows. A nonspecific band between 50 and 60 kDa is visible in some lanes and represents Ig heavy chain variably retained on the beads.

mains as well as a sequence motif (db1 homology domain) found in various proteins known to function as guanine nucleotide exchange factors (GEF) for Ras and Ras-related proteins (28–30). On this basis, it has been suggested that Vav represents a new class of signaling substrates, the activation of which may provide a mechanism for coupling cell surface receptors to Ras (22–24). However, at present the precise functions for Vav are unclear, as Vav has been shown to act as a Ras GEF in T and B lymphocytes (31, 32), but appears to induce NIH3T3 transformation by mechanisms independent of Ras activation (33, 34). While the substrates for Vav GEF activity remain to be defined, the cumulative data concerning Vav, including its potential for oncogenic activation (35), suggest that the modulation of Vav signal transducing functions(s) represents another possible mechanism whereby PTP1C might influence the development and functions of multiple hemopoietic cell lineages.

To investigate this possibility, we evaluated the capacity of PTP1C to interact with Vav in resting and activated mast cells and T lymphocytes. As reported here, the results of this analysis reveal the association of PTP1C protein and tyrosine phosphatase activity with Vav and indicate that this interaction increases following mitogenic stimulation of these cells and coincident with increases in tyrosine phosphorylation of both Vav and PTP1C. The data also implicate both the SH2 and SH3 domains of Vav in mediating the association of this protein with PTP1C. Lastly, while a major portion of total intracellular

PTP1C activity appears to be contained in Vav-PTP1C complexes, the results of this study demonstrate that PTP1C also binds to both Grb2 adaptor and mSos1 GEF proteins. Together, these results suggest that PTP1C effects on hemopoietic cell growth and development may be realized at least in part through modulation of the signaling events linking receptor stimulation to the activation of Ras or Ras-related proteins.

MATERIALS AND METHODS

Reagents—Polyclonal anti-PTP1C and anti-Syp antibodies were generated in rabbits immunized with GST-PTP1C SH2 domain fusion proteins as described previously (5, 9). The monoclonal anti-phosphotyrosine 4G10 and anti-Vav antibodies as well as rabbit polyclonal anti-murine son-of-sevenless 1 (mSos1) antibodies were obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Rabbit polyclonal antibody specific to Grb2 and a synthetic peptide corresponding to residues 577–590 of the mouse Vav protein were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Steel factor (SF) was obtained from Genzyme (Cambridge, MA), and concanavalin A (Con A) as well as all chemicals for immunoblotting/immunoprecipitation analyses were obtained from Sigma.

Cells and Cell Lines—Fresh splenic cell suspensions used in this work were prepared by standard procedures from C57BL/6J-*me^v/me^v* and $+/+$ and C3HeBFeJ-*me/me* and $+/+$ mice derived from breeding stock maintained at the Samuel Lunenfeld Research Institute, Mount Sinai Hospital. The murine EL4 T lymphoma and P815 mastocytoma lines were obtained from Dr. C. Paige and ATCC (TIB64), respectively, and were maintained at 37°C in Opti-MEM (Life Technologies, Inc.) containing $100 \mu\text{g}/\text{ml}$ penicillin/streptomycin and 10% fetal calf serum (Life Technologies, Inc.). As controls for some experiments, we also used

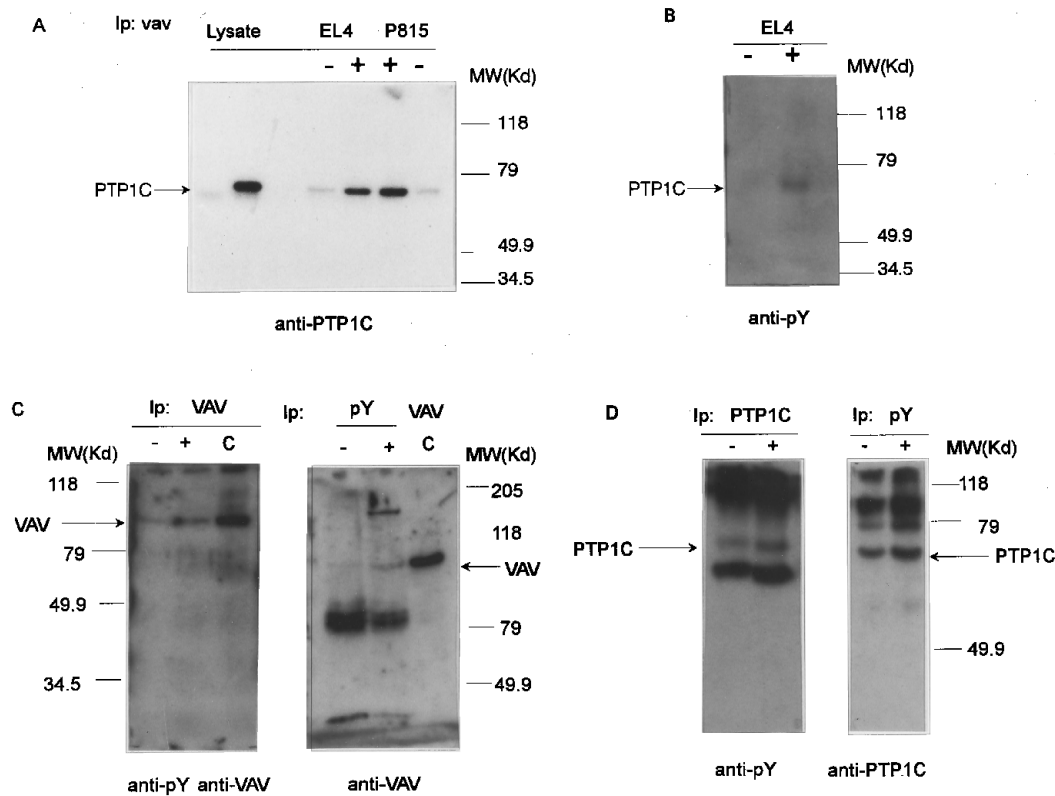


FIG. 2. Increases in PTP1C-Vav association and tyrosine phosphorylation following cell stimulation. A, cell lysates were prepared from unstimulated (–) or ConA (20 μ g/ml)-treated (+) EL4 cells and unstimulated (–) or steel factor (100 ng/ml)-treated P815 cells, and 800 μ g of lysate proteins were immunoprecipitated with anti-Vav antibodies. Lysates were also prepared from 10^8 B16 melanoma cells stably transfected with either pCMV4Neo vector alone (far left lane) or pCMV4Neo ligated to the full-length PTP1C cDNA (second lane from the left), and the lysates and immunoprecipitated proteins were resolved by SDS-PAGE and immunoblotted with anti-PTP1C antibody. B, cell lysates were prepared from unstimulated (–) and Con A-treated (+) EL4 cells and 800 μ g of lysate proteins were immunoprecipitated with anti-PTP1C antibody, resolved over SDS-PAGE, and immunoblotted with the 4G10 anti-phosphotyrosine (pY) antibody. C, cell lysate proteins (500 μ g) prepared from unstimulated (–) and steel factor-treated (+) P815 cells were immunoprecipitated with anti-Vav (left panel) or anti-phosphotyrosine (right panel) antibodies, resolved over SDS-PAGE, and immunoblotted with anti-phosphotyrosine (left panel) or anti-Vav (right panel) antibodies. As a control (C), Vav immunoprecipitates prepared from P815 cell lysates (800 μ g) were immunoblotted with anti-Vav antibody. D, cell lysates were prepared from unstimulated (–) and steel factor-treated (+) P815 cells, and 800 μ g of lysate proteins were immunoprecipitated with anti-PTP1C (left panel) or anti-phosphotyrosine (right panel) antibodies, resolved over SDS-PAGE, and immunoblotted with anti-phosphotyrosine (left panel) or anti-PTP1C (right panel) antibodies. The positions of molecular mass standards are indicated in all four panels; arrows indicate the positions of PTP1C and Vav.

B16 melanoma cells transfected with the vector pCMV4Neo or alternatively with a construct containing the full-length PTP1C cDNA subcloned into the pCMV4Neo vector (provided by Dr. B. Chan). These latter lines were maintained under the same culture conditions as described above except for the addition of 2 mg/ml Geneticin (Life Technologies, Inc.) to the culture medium. For cell stimulations, cells were cultured in Opti-MEM containing 0.5% fetal calf serum for 17 h, washed, and then cultured for 10 min in the presence of 20 μ g/ml ConA (EL4 and splenic cells) or for 5 min in the presence of 100 ng/ml steel factor (P815 cells).

Generation of GST-Vav Fusion Proteins—Glutathione S-transferase (GST)-Vav fusion proteins were generated by subcloning polymerase chain reaction-amplified murine Vav sequences into pGEX2T. The amplified fragments (illustrated in Fig. 3A) subcloned into this expression plasmid include the Vav SH2 domain alone (amino acids 670–765), the Vav SH2 and C-terminal SH3 domains (amino acids 670–942), and the Vav N-terminal SH3, SH2, and C-terminal SH3 domains (amino acids 611–942). GST-Vav expression plasmids were transfected into *Escherichia coli*. GST-Vav fusion proteins were purified from isopropyl- β -D-galactopyranoside-induced bacteria with glutathione-conjugated Sepharose beads (Pharmacia, Baie d'Urf 130, Quebec).

Immunoblotting Analysis—Protein lysates were prepared by resuspending 10^7 – 10^8 resting or mitogen-treated splenic, EL4, and P815 cells in 1 ml of lysis buffer (phosphate-buffered saline containing 1% Triton X-100, 1% Tween, 1 mM sodium orthovanadate, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin, and 0.001 mM dithiothreitol). 100 μ g of cell lysate protein was electrophoresed through 10% SDS-polyacrylamide gels and electroblotted onto nitrocellulose, and the blots then were incubated overnight at 4 $^{\circ}$ C in 10 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% Tween 20 (TBST) containing 5% skim milk. The proteins were then detected by

incubating blots for 2 h at room temperature with primary antibodies in TBST followed by 125 I-protein A (Dupont, Canada). Blots were then washed with TBST and exposed to Kodak XAR film at -70° C. For immunoprecipitations, cell lysates were prepared from 10^8 resting or mitogen-treated splenic, EL4, and P815 cells, and the lysates were clarified by centrifugation for 10 min at $10,000 \times g$ at 4 $^{\circ}$ C. 500–2000 μ g of cell lysate protein was incubated for 2 h at 4 $^{\circ}$ C with selected antibodies and then with 100 μ l of protein A-Sepharose (Pharmacia) for 10 min at 4 $^{\circ}$ C. The immune complexes were collected by centrifugation, washed three times with lysis buffer, boiled for 5 min in SDS-sample buffer, and then subjected to electrophoresis and immunoblotting as described above.

In Vitro Binding Assays—To evaluate PTP1C binding to GST-Vav fusion proteins, protein lysates prepared from 10^8 steel factor-treated P815 cell lysates were incubated at 4 $^{\circ}$ C for 2 h with 5 μ g of fusion protein immobilized on glutathione-Sepharose beads. After several washes in lysis buffer, complexes were resuspended in sample buffer, boiled, and analyzed by SDS-PAGE and immunoblotting with anti-PTP1C antibody and 125 I-protein A.

Assays of Phosphatase Activity—To assay PTP1C and Vav-associated phosphatase activities, PTP1C and Vav were immunoprecipitated as described above from 300, 600, or 900 μ g of cell lysate proteins prepared from 10^7 – 10^8 P815 cells incubated for 5 min in medium containing 100 ng/ml steel factor. Immunoprecipitates were washed three times in lysis buffer and then incubated at 37 $^{\circ}$ C for 4 h in 200 μ l of phosphatase buffer (62 mM Hepes, pH 7.5, 6.25 mM EDTA, 12.5 mM dithiothreitol) containing 2 mM *p*-nitrophenyl phosphate (Sigma). Reactions were terminated by addition of 1 ml of 0.2 M NaOH, and absorbance was measured at 410 nm. Alternatively, tyrosine phosphatase activity was measured in Vav and Syp immunoprecipitates prepared from cell ly-

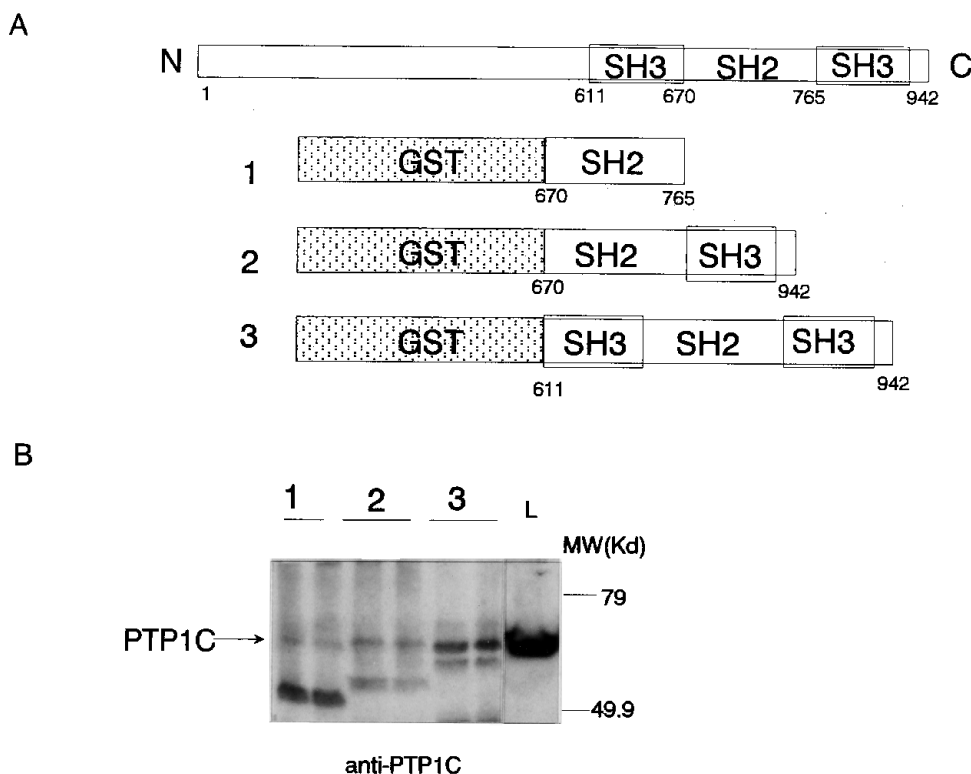


FIG. 3. Association of Vav SH2/SH3 domains with activated PTP1C. *A*, schematic showing Vav sequences present in the three GST-Vav fusion proteins used for *in vitro* binding assays. Numbers below each construct refer to amino acid positions of domain boundaries. *B*, cell lysates were prepared from 10^8 steel factor-stimulated P815 cells and incubated for 2 h at 4 °C with 5 μ g of purified GST-fusion protein immobilized on glutathione-Sepharose beads. Complexes as well as lysate (*L*) alone (*i.e.* no GST-fusion protein added) were electrophoresed through SDS-PAGE and subjected to immunoblotting with anti-PTP1C antibody. Numbers at the top represent the GST-Vav expression protein used in duplicate samples. Molecular size markers are indicated on the right, and the position of PTP1C is shown on the left.

sates of 10^8 motheaten or congenic wild-type ConA (20 ng/ml)-treated splenic cells. For this assay, the immunoprecipitated proteins were incubated at 37 °C for 2 h in 25 μ l of 10 mM Tris HCl, pH 7.4, containing 7.5 mM tyrosine phosphopeptide (RRLIEDAEPYAARG), and the reaction was terminated by addition of Malachite Green solution (UBI) as described previously (36). Phosphate release was measured after 15 min by evaluating absorbance at 605 nm.

RESULTS AND DISCUSSION

PTP1C Interacts with Vav in Resting P815 Cells—To investigate the possible association of PTP1C with Vav in unstimulated hemopoietic cells, Vav immunoprecipitates prepared from P815 mastocytoma cell lines and from *me*, *me^v*, and wild-type control splenic cells were examined by immunoblotting analysis for the presence of PTP1C. As shown in Fig. 1, the ~70-kDa PTP1C protein was coprecipitated with Vav from both P815 cells (Fig. 1A) and resting normal murine splenocytes (Fig. 1C). This association was detected both by immunoblotting anti-Vav immunoprecipitates with anti-PTP1C antibody and, conversely, by immunoblotting anti-PTP1C immunoprecipitates with anti-Vav antibody (Fig. 1A and B). Moreover, the capacity of PTP1C to interact with Vav in P815 cells was observed to be diminished markedly by preincubation of the P815 lysates with a synthetic peptide representing a 13-amino-acid segment of the Vav protein (Fig. 1A). As anticipated, no PTP1C was detected in Vav immunoprecipitates from *me* splenic cells (Fig. 1C) which have been shown to lack PTP1C protein (9). By contrast, while the phosphatase domain mutations found in *me^v* PTP1C proteins severely reduce this enzyme's catalytic activity, these mutant proteins retain the capacity to interact with Vav (Fig. 1C).

The data shown in Fig. 1A also reveal isolated murine splenocytes to express two PTP1C species (~67 and 70 kDa, respectively), only one of which (the latter species) is detectable

in P815 cells. The existence of PTP1C isoforms has been observed previously in other hemopoietic cell populations (9) and, based on sequence analysis of PTP1C transcript, ascribed to the alternative splicing of a 39-amino-acid segment within the PTP1C C-terminal SH2 domain (7). The functional significance of PTP1C SH2 domain variants is not known, but the expression of only one PTP1C species in P815 cells is consistent with previously reported data indicating the two species to be expressed variably in different hemopoietic and epithelial lineages (9, 37). Moreover, based on the exclusive detection of the higher molecular weight PTP1C species in Vav immunoprecipitates from murine splenic cells (Fig. 1C), it appears that Vav may selectively interact with this single PTP1C variant. While further studies are required to address this issue, the data shown here reveal the capacity of PTP1C to associate with Vav in both mast and unseparated splenic cells and suggest that the PTP1C sequences which mediate Vav binding in resting cells map to regions flanking the site of the *me^v* phosphatase domain mutation.

PTP1C Binding to Vav Increases following Mitogenic Stimulation and Appears to Be Mediated through the Vav SH2 Domain—It has been previously reported that PTP1C association with both the *c-kit* and IL-3 receptors is markedly increased following receptor engagement (10, 11). To determine whether PTP1C binding to Vav is also increased following cell stimulation, Vav immunoprecipitates were prepared from resting, ConA-treated EL4 and steel factor-treated P815 cells, and the coprecipitation of PTP1C was assessed by immunoblotting analysis. As shown in Fig. 2A, stimulation of both EL4 and P815 cells induced marked increases in the association of PTP1C with Vav. This result cannot be ascribed to ConA/steel factor-driven increases in expression of these proteins, as levels

of PTP1C and Vav were not appreciably different in resting *versus* stimulated cells (data not shown). By contrast, steel factor and ConA treatment induced marked increases in Vav tyrosine phosphorylation in P815 (Fig. 2C) and EL4 (data not shown) cells, respectively. PTP1C tyrosine phosphorylation was also increased in association with stimulation of these cells (Fig. 2, B and D). These findings are consistent with previous data revealing the induction of Vav (22–27) and PTP1C (13, 38, 39) tyrosine phosphorylation following stimulation of a variety of cell surface receptors and suggest that the enhanced association of PTP1C with Vav in activated cells is mediated through an SH2 domain-phosphotyrosine interaction.

In view of these findings, as well as previous data showing that Vav association with another signaling effector in activated T cells, the protein tyrosine kinase ZAP70, is mediated through binding of the Vav SH2 domain to phosphotyrosine site(s) on ZAP70 (40), the contribution of the Vav SH2 domain to Vav-PTP1C interaction was investigated. To this end, GST fusion proteins containing the Vav SH2 domain alone and the Vav SH2 domain combined with the carboxyl-terminal or both Vav SH3 domains (Fig. 3A) were coupled to glutathione-Sepharose, incubated with steel factor-treated P815 cells, and evaluated for PTP1C binding by immunoblotting with anti-PTP1C antibody. As shown in Fig. 3B, the results of this *in vitro* analysis revealed PTP1C binding with the fusion protein containing the Vav SH2 domain alone, but PTP1C binding was observed considerably increased with the fusion proteins containing an SH2 and SH3 domain and even more increased with the fusion protein containing both Vav SH3 domains. These results indicate the capacity of the Vav SH2 domain to interact with PTP1C in activated cells, and, as has been demonstrated previously with respect to the SH2 domain-mediated intramolecular repression of Src activity (41), the data also suggest that optimal binding of these molecules requires the Vav SH3 domains as well. However, these results do not preclude the possibility that the PTP1C SH2 domains and/or other sites within the Vav protein contribute to the interaction of these proteins.

Detection of Vav-associated Tyrosine Phosphatase Activity—To evaluate the potential biologic relevance of PTP1C association with Vav, Vav was immunoprecipitated from steel factor-treated P815 cells and the immune complexes were assessed for associated phosphatase activity. As evaluated by the dephosphorylation of *p*-nitrophenol phosphate substrate, phosphatase activity was clearly detected in these immunoprecipitates, the levels of activity increasing linearly in proportion to the amount of cell lysate protein (Fig. 4A). To evaluate the extent to which PTP1C contributes to Vav-associated phosphatase activity, this experiment was repeated using ConA-treated splenic cells from *me* mice. As shown in Fig. 4A, the level of Vav-coprecipitated phosphatase activity in these PTP1C-deficient cells was negligible and unaltered by the use of increasing amounts of cell lysate protein. These findings suggest that PTP1C accounts for the majority of Vav-associated phosphatase activity detected in steel factor-treated P815 cells and thus imply that Vav or Vav-associated signaling molecules may represent targets for PTP1C-induced tyrosine dephosphorylation.

To extend these data, Vav immunoprecipitates from ConA-treated normal *me* and *me^v* splenic cells were also assessed for their capacity to dephosphorylate a tyrosine-phosphorylated synthetic peptide. As is consistent with the contention that PTP1C accounts for the majority of Vav-associated phosphatase activity, levels of tyrosine phosphatase activity detected in Vav immunoprecipitates from *me* and *me^v* splenic cells were dramatically reduced relative to those observed in splenic cell

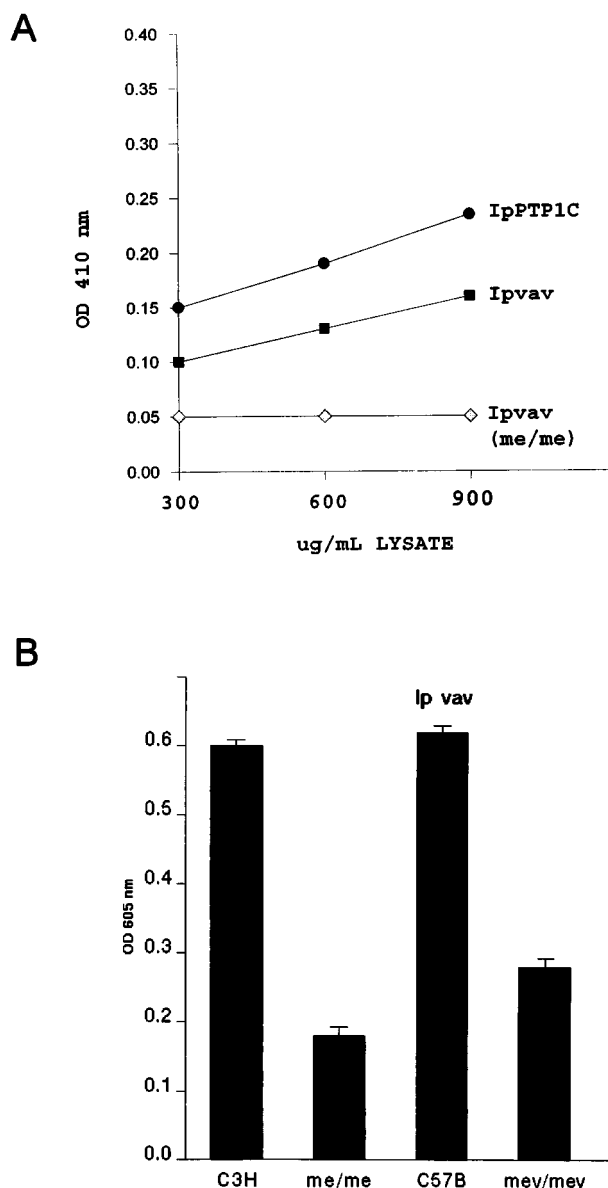


FIG. 4. Identification of tyrosine phosphatase activity in Vav immunoprecipitates from stimulated splenic and P815 cells. A, cell lysates were prepared from steel factor-treated P815 cells and from ConA-treated splenic cells obtained from *me* mice. Aliquots of 300, 600, and 900 μ g of lysate proteins were immunoprecipitated from P815 cells with anti-PTP1C antibody (*IpPTP1C*) and from both P815 and *me* splenic cells with anti-Vav antibodies (*IpVav* and *IpVav (me/me)*, respectively). The immunoprecipitates were incubated with 2 mM *p*-nitrophenol phosphate at 37 °C for 4 h, and, after addition of NaOH, absorbance was measured at 410 nm. The results shown are representative of three independent experiments. B, cell lysates were prepared from 10^8 ConA-stimulated C3HeBFeJ wild-type (C3H) and *me* (*me/me*) and C57BL/6J wild-type (C57B) and *me^v* (*me^v/me^v*) splenic cells, and the lysate proteins (800 μ g) were immunoprecipitated with anti-Vav antibody. The immunoprecipitates were incubated at 37 °C for 2 h with a synthetic tyrosine-phosphorylated peptide as described under "Materials and Methods," and the reaction was terminated by addition of Malachite Green. The amount of phosphate released was determined spectrophotometrically by measuring absorbance at 605 nm. The results shown are representative of two independent experiments, and the bars indicate standard deviations for a single experiment performed in duplicate.

Vav immunoprecipitates from congenic wild-type mice (Fig. 4B). By contrast, Syp-precipitable tyrosine phosphatase activity was essentially the same in *me^v* and wild-type splenic cells (data not shown). Together, these data indicate the association of Vav with tyrosine phosphatase activity and strongly suggest

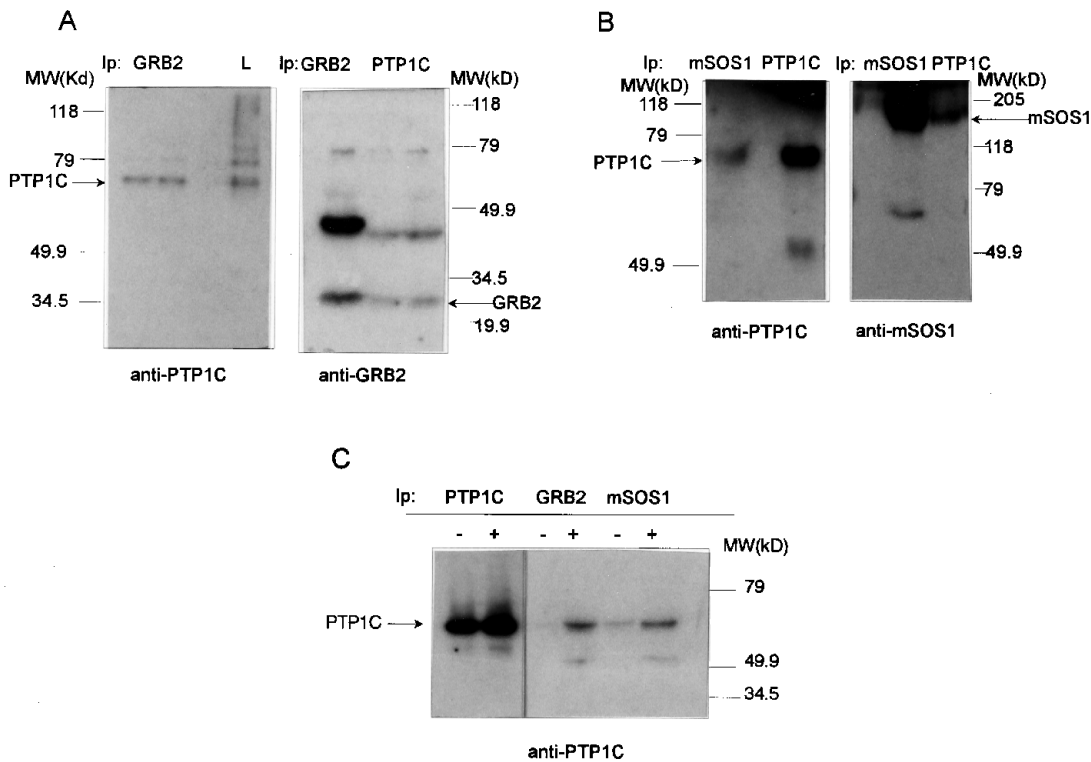


FIG. 5. **Association of PTP1C with Grb2 and mSos1 in P815 cells.** A, cell lysates were prepared from unstimulated P815 cells and 2000 μ g of lysate protein immunoprecipitated with anti-Grb2 (left panel) or anti-PTP1C (right panel) antibodies. Duplicate samples of the precipitated proteins as well as 500 μ g of lysate protein alone (L, left panel) or 1500 μ g of lysate proteins immunoprecipitated with anti-Grb2 antibody (Ip:GRB2, right panel) were electrophoresed through SDS-PAGE and immunoblotted with anti-PTP1C (left panel) or anti-Grb2 (right panel) antibodies. B, cell lysates were prepared from unstimulated P815 cells, and 2000 μ g of lysate proteins were immunoprecipitated with either anti-mSos1 or anti-PTP1C antibodies (as shown on top of each panel), subjected to SDS-PAGE, and then immunoblotted with anti-PTP1C (left panel) or anti-mSos1 (right panel) antibodies. C, cell lysates were prepared from unstimulated (–) and steel factor-treated (+) P815 cells, and 1000 μ g of lysate proteins were immunoprecipitated with anti-PTP1C, anti-Grb2, or anti-mSos1 antibodies as indicated, electrophoresed through SDS-PAGE, and immunoblotted with anti-PTP1C antibody. For all panels, the positions of molecular mass markers are shown on the side, and arrows indicate the positions of PTP1C, mSos1, and Grb2.

that this activity is engendered by PTP1C. Based on the relative levels of phosphatase activity contained in Vav *versus* PTP1C immunoprecipitates from stimulated P815 cells (Fig. 4A), it also appears that a considerable proportion of cellular PTP1C activity is associated with Vav, an observation which is consistent with previous data indicating that PTP1C-ligand binding substantially enhances PTP1C catalytic function (42). By inference, these findings are highly suggestive of a critical role for PTP1C in modulating the signal transducing functions of Vav and/or Vav-associated proteins.

Association of PTP1C with Grb2 and mSos1 in P815 Cells—While the precise relationship between Ras activation and Vav-mediated transformation remains unclear, recent data suggest that Ras proteins and Vav cooperate in a synergistic fashion to induce cellular transformation (34). The potential relevance of Vav to Ras-related signaling events has also been suggested by the observed capacity of Vav to associate physically with the Grb2 protein (43), a ubiquitously expressed molecule known to interact via its SH3 domains with proline-rich motifs in the Ras GEF mSos1 and via its SH2 domain to phosphotyrosine residues in activated growth factor receptors (44–46). In view of these findings and the current data revealing the association of PTP1C with Vav, we next explored the possibility that PTP1C might also interact with the Grb2-mSos1 complex in hemopoietic cells. To this end, Grb2 and mSos1 or, alternatively, PTP1C immunoprecipitates were prepared from resting and steel factor-treated P815 cells and subjected to immunoblotting analysis with anti-PTP1C or anti-Grb2/mSos1 antibodies, respectively. As shown in Fig. 5, the results of this analysis reveal the capacity of PTP1C to bind to both Grb2 (Fig. 5A) and mSos1

(Fig. 5B) in unstimulated cells. As the interaction of Grb2 with Vav has also been detected in unstimulated T cells (43), it appears that PTP1C, Vav, and the Grb2-mSos1 molecules may associate with one another as a multimeric complex in resting hemopoietic cells. PTP1C interactions with Grb2 and mSos1 were also examined in steel factor-treated cells, and, as observed with respect to PTP1C binding to Vav, interactions between this phosphatase and both Grb2 and mSos1 were increased, following cell activation (Fig. 5C). These findings are again consistent with the contention that PTP1C and Vav interactions modulate receptor-evoked mitogenic signaling cascades and suggest that this modulatory effect also involves the association of these proteins with Grb2-mSos1. While the structural basis for the amalgamation of these four signaling effectors requires further definition, recent data revealing the capacity of the Grb2 carboxyl-terminal SH3 domain to physically associate with the Vav amino-terminal SH3 domain (47) together with data implicating the Grb2 amino-terminal SH3 domain in mSos1 binding (45) provide some indication as to the mode of Grb2-mSos1 association with PTP1C-Vav complexes. In addition, the localization of a major site for PTP1C tyrosine phosphorylation (Tyr-538) within a sequence (pYGNX) representing a consensus sequence (pYXNX) for Grb2 SH2 domain binding (38, 39, 48) suggests a role for Grb2 SH2 domain interaction with PTP1C phosphotyrosine in the genesis of this multimeric complex. In this context, PTP1C may act as an adaptor linking activated receptors to Grb2-mSos1, a role already demonstrated for the ubiquitously expressed Syp PTP in relation to the *c-kit* and platelet-derived growth factor receptors (49, 50). Whether or not PTP1C plays this latter role in

relation to Grb2-mSos1 and Vav, the interaction of this phosphatase with these specific cytosolic signaling molecules suggests a critical influence of this phosphatase on the signaling cascades linking hemopoietic cell surface receptors to Ras activation.

In summary, we have shown that PTP1C associates with Vav, Grb2, and mSos1, three cytosolic molecules expressed broadly among hemopoietic cells and implicated in the activation of Ras or Ras-related signaling pathways. The capacity of PTP1C to interact with and potentially modulate these signaling proteins strongly suggests that PTP1C effects on hemopoietic cell differentiation and growth are realized at least in part through the regulation of Ras and/or Ras-related proteins. Similarly, the association of Vav with PTP1C protein and phosphatase activity implies a role for PTP1C in modulating Vav-induced transformation events. The definition of the structural basis for and physiologic relevance of PTP1C associations with these signaling effectors thus represents a promising avenue toward elucidating the intracellular events regulating downstream transmission of receptor-evoked activation signals in hemopoietic cells.

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