Signaling Capacity of the T Cell Antigen Receptor Is Negatively Regulated by the PTP1C Tyrosine Phosphatase

By Giovanni Pani,[*‡] Klaus-Dieter Fischer,[‡] Irena Mlinaric-Rascan,[*‡] and Katherine A. Siminovitch[*‡]

From the Departments of *Medicine, Immunology, and ‡Molecular and Medical Genetics, University of Toronto, and the Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada, M5G 1X5

Summary

The association of PTP1C deficiency with the multiplicity of lymphoid cell abnormalities manifested by motheaten (me) and viable motheaten (me^v) mice suggests a pivotal role for this tyrosine phosphatase in the regulation of lymphocyte differentiation and function. To delineate the relevance of PTP1C to T cell physiology, we have examined me and me^v T cells with regards to their capacity to transduce activating signals through the T cell antigen receptor (TCR). Although thymocyte maturation appeared normal in the mutant mice, both thymocytes and peripheral T cells from these animals exhibited proliferative responses to TCR stimulation that were markedly increased relative to those elicited in normal cells. Compared to normal thymocytes, PTP1C-deficient thymocytes also showed increased constitutive tyrosine phosphorylation of the TCR complex and enhanced and prolonged TCR-induced tyrosine phosphorylation of the TCR-ζ and CD3-ε, as well as a number of cytosolic proteins, most notably a 38-kD phosphoprotein found to associate with the Grb2 adaptor SH2 domain in activated thymocytes. These latter phosphoproteins also associated with the Vav guanine nucleotide exchange factor upon TCR ligation, and were dephosphorylated by recombinant PTP1C in vitro. In conjunction with the finding of PTP1C–TCR association in unstimulated normal thymocytes, these results reveal the capacity of PTP1C to interact with and likely dephosphorylate resting and activated TCR complex components, as well as more distal signaling effectors that are normally recruited to the Vav and Grb2 SH2 domains after TCR stimulation. These data therefore strongly implicate PTP1C in the downregulation of TCR signaling capacity and, taken together with the aberrant prolongation of TCR-induced, mitogen-associated kinase (MAPK) activation observed in PTP1C-deficient thymocytes, these findings suggest that the inhibitory influence of PTP1C on TCR signal relay is realized through its effects on both the TCR complex and downstream signaling elements that couple the activated antigen receptor to the Ras/MAPK response pathway.

The intracellular events linking TCR engagement to a physiologic response involve a multiplicity of molecular interactions, the specificity and sequelae of which depend on the cell's developmental stage and exposure to costimulatory signals. Although the precise amalgam of molecular events that translate TCR engagement to specific types of cell behavior remain unclear, a wealth of information has accrued with respect to the general molecular strategies used to couple the stimulated TCR to the nucleus. As for other growth receptors, the downstream propagation of activation signals from the TCR is known to be critically dependent on tyrosine phosphorylation, both TCR components and numerous cytosolic signaling effectors becoming rapidly phosphorylated after TCR stimulation (1). In contrast to many growth factors, however, the signaling elements within the TCR complex lack intrinsic enzymatic activity, and their tyrosine phosphorylation is thus achieved through interactions with nonreceptor protein tyrosine kinases (PTKs)[1], most notably ZAP-70, syk, and the src-related lck and fyn proteins (2, 3). The effects of these PTKs on TCR signaling capacity primarily reflects their phosphorylation of tyrosine residues mapping within highly conserved domains (tyrosine-based activation motifs) of the TCR–CD3-ε, ζ and η subunits with the consequent generation of high affinity binding sites for the recruitment of SH2 domain–containing effectors into the activation path-

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[1] Abbreviations used in this paper: BCR, antigen receptor on B cells; GEF, guanine nucleotide exchange factors; GST, glutathione S-transferase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; PLC, phospholipase C; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase.

way (4, 5). One such signaling effector is the ZAP-70 PTK, which upon TCR ligation, binds via its SH2 domains to tyrosine-phosphorylated TCR and manifests a marked increase in catalytic activity thought to be responsible for the phosphorylation and activation of phospholipase C (PLC) γ1 and consequent induction of the phosphoinositide second-messenger pathway (6–8). Along similar lines, PTK-mediated tyrosine phosphorylation of TCR subunits also results in recruitment of Grb2 and possibly other adaptor proteins, and these in turn serve to link the activated receptor to guanine nucleotide exchange factors (GEFs), such as mSos, and thus to the activation of Ras and downstream mitogen-activated protein (MAP) kinases (9, 10). While TCR signaling capacity clearly reflects a host of other molecular interactions, these examples illustrate the pivotal role for PTK-induced phosphorylation in the coupling of TCR engagement to a cell response.

In contrast to the extensive data that is available concerning PTK involvement in TCR-driven signaling events, much less is known with respect to the contribution of protein tyrosine phosphatases (PTPs) to the modulation of antigen receptor function. On the basis of considerable biochemical and genetic data, the single PTP that has been widely studied in relation to T cell signaling, CD45, appears to exert a primarily positive influence on the TCR signaling capacity activity of this enzyme, apparently promoting T cell development and activation (11–13). By contrast, the observed capacity of phosphatase inhibitors to enhance or even induce intracellular molecular events normally elicited by TCR ligation (14) suggests that at least some PTPs act in the downregulation of TCR-evoked signaling events. Thus, by providing the biochemical means to counterbalance PTK effects and terminate receptor-initiated signal transmission, PTPs potentially exert a key inhibitory influence on T cell signaling circuitry.

Among the PTPs identified to date, the cytosolic enzyme PTP1C (SHP/HCP/SHPTP1) represents a signaling effector that is particularly likely to be involved in the negative regulation of TCR signaling capacity. This SH2 domain-containing enzyme has, for example, been shown to not only physically associate with the antigen receptor on B cells (BCR), but to downregulate BCR-transduced signals evoking either the activation of mature B cells or the deletion/negative selection of autoreactive precursor B cells (15, 16). PTP1C interactions with the CD22 and FcγRIIB receptors on B cells also appear to culminate in the downregulation of BCR-triggered activation signals (17, 18). These findings, together with recent data revealing a negative regulatory influence of PTP1C on the signaling functions of the erythropoietin and IL-3 receptors (19, 20), suggest that PTP1C may also exert inhibitory effects on the signaling cascades elicited by TCR engagement. To address this possibility, we have examined TCR signaling capacity in thymocytes from motheaten (*me*) and viable motheaten (*me^v*) mice, animals in which PTP1C gene mutations result in the absence of catalytically active enzyme (21, 22). While overt defects in thymocyte maturation were not de-

tected in the mutant mice, the induction of proliferation, protein tyrosine phosphorylation, and MAP kinase activity after TCR stimulation was markedly increased in *me* and *me^v* thymocytes relative to cells from congenic wild-type mice. Among the phosphoproteins showing increased levels and duration of tyrosine phosphorylation in the TCR-stimulated mutant cells were a number of species that associate with PTP1C and/or the Vav SH2 domain, and that are subject to PTP1C tyrosine dephosphorylation in vitro. These latter proteins were found to include several components of the TCR complex, as well as a 38-kD species (pp38) that also associates strongly with the SH2 domain of the Grb2 adaptor protein after TCR ligation. Together, these findings indicate that PTP1C is involved in the downregulation of TCR-initiated signal transduction pathways, and suggest that PTP1C subserves this inhibitory role through participation in a multimeric signaling complex comprised of PTP1C, Vav, Grb2, and various phosphoproteins such as pp38 and phosphorylated TCR subunits, which appear to represent PTP1C substrates. These findings therefore reveal the negative influence of PTP1C on T cell signaling to be realized through PTP1C effects on both the TCR complex and more downstream signaling effectors that couple the TCR to Ras/MAP kinase activation.

Materials and Methods

Mice. Mice for this work were obtained by mating C3HeBFeJ *me*/+ and +/+ and C57BL/6J *me^v*/+ and +/+ breeding pairs derived from stock maintained at the Samuel Lunenfeld Research Institute, Mount Sinai Hospital (Toronto, Ontario). All mice used in this study were 2–3 wk old.

Reagents. Antibodies used for these studies include FITC-conjugated anti-CD4, PE-conjugated anti-CD8, and biotin-conjugated monoclonal hamster anti–mouse TCR (β chain) antibodies (Pharmigen, San Diego, CA); a monoclonal rat anti–mouse CD3 antibody provided by Dr. G. Mills (M.D. Anderson Cancer Center, Houston, TX); a monoclonal rat anti–mouse CD5 antibody (Pharmigen); rabbit polyclonal anti-Vav, anti-Grb2, and anti-ERK2(MAPK) antibodies (Santa Cruz Laboratories, Santa Cruz, CA); 4G10 monoclonal anti-phosphotyrosine antibody (Upstate Biotechnology Inc., Lake Placid, NY); rabbit polyclonal PTP1C SH2 domain antibody produced in our lab (21); a rabbit polyclonal anti–mouse TCR-ζ provided by Dr. L. Samelson (NIH, Bethesda, MD); monoclonal anti–mouse CD3-ε provided by Dr. A. Singer (NIH, Bethesda, MD); and mouse IgG (Sigma Chemical Co., St. Louis, MO). The glutathione S-transferase (GST) fusion proteins used in this study were derived by subcloning the following cDNA or PCR-amplified fragments into pGEX2T: the full-length murine PTP1C cDNA (PTP-1C/GST), a full-length murine PTP1C cDNA containing a Cys453→Ser mutation known to abrogate catalytic activity, provided by Dr. W. Muller, (McMaster University, Hamilton, Ontario) (C/SPTP1C/GST), and the Vav SH2 domain (amino acids 663–776) alone (Vav SH2/GST) and the Grb2 SH2 domain (amino acids 60–158) alone (Grb2 SH2/GST), provided by Dr. M. Rozakis-Adcock (Toronto, Ontario) (23). These GST expression plasmids were transfected into *Escherichia coli* JM101, and the fusion proteins were purified from bacterial lysates after isopropyl β-D-thiogalactopyranoside induction. OVA was obtained

from U.S. Biochemical Corp. (Cleveland, OH) and Con A, PMA, and all chemicals for immunoblotting/immunoprecipitation analyses were purchased from Sigma Chemical Co.

Cells and Cell Lines. Single-cell suspensions were prepared from *me*, *me^v*, and wild-type congenic (+/+) lymph nodes and thymus, subjected to erythrocyte lysis by 2-min incubation in 0.8% ammonium chloride and the viability was determined by staining in 0.4% trypan blue (Sigma Chemical Co.). The murine EL4 thymoma (TIB 39; American Type Culture Collection, Rockville, MD) was maintained at 37°C in complete DMEM medium (GIBCO BRL, Gaithersburg, MD) containing 10% FCS (GIBCO BRL), 50 μm 2-ME, 2 mm L-glutamine, and nonessential amino acids. Cells were harvested in log growth phase.

Fluorescence Staining (FACS)®. Single-cell suspensions prepared from *me*, *me^v*, and wild-type congenic thymus were assessed for CD4, CD8, and TCR expression by immunofluorescent analysis, as described previously (24). Briefly, erythrocyte-depleted thymic single-cell suspensions (10^6 cells) were incubated for 30 min at 4°C with anti–CD4-FITC, anti–CD8-PE, and biotinylated anti-TCR antibodies. Cells were then washed and incubated for a further 30 min at 4°C with Tricolor Avidin (Caltag Laboratories, San Francisco, CA), and were then washed and resuspended in staining solution. All samples were then examined by fluorescence cytometry using a FACScan® cell analyzer (Becton Dickinson & Co., Mountain View, CA).

Proliferation Assays. Single-cell suspensions of thymocytes harvested from *me*, *me^v*, and wild-type littermates were cultured in 96-well flat-bottom microtiter plates (5 × 10^5 cells/100 μl per well) in α-MEM (GIBCO BRL) containing 10% heat-inactivated FCS, 50 μM 2-ME, and penicillin/streptomycin. For proliferation studies, cells were cultured directly with either 2.2 μg Con A or 50 ng/ml PMA plus ionomycin (1 μM), or in the presence of 25 U/ml IL-2 (Sigma) in anti-CD3 antibody–coated culture plates prepared by overnight incubation of the plates at 4°C with anti-CD3 antibody dissolved in 0.05 M Tris-HCl, pH 9.2. Cells were cultured for 48 h at 37°C, 5% CO_2, and pulsed with [^{3}H]thymidine (1 μCi/well; Amersham International, Amersham, UK) 6 h before terminating incubation, and incorporated radioactivity was measured using an automated β liquid scintillation counter. For OVA proliferation assays, groups of *me^v* mice and normal littermates were treated by footpad injection of 50 or 100 μg, respectively, OVA in CFA (GIBCO BRL), or CFA alone, as previously described (24). Animals were killed 7 d after immunization, and their popliteal, inguinal, and paraaortal lymph nodes were harvested and used to prepare single-cell suspensions. To reduce effects of differences between *me^v* and normal accessory cells, cells obtained from two OVA-primed animals from the same group (i.e., *me^v* or normal) were pooled and mixed with equal numbers of cells obtained from the CFA-only–treated mice of the other group. Cells were then cultured (5 × 10^5 cells/well) for 72 h in α-MEM medium (as described above) in the presence or absence of varying amounts of OVA (ranging from 300 ng/ml to 3 mg/ml), and radiolabeled thymidine incorporation during the last 6 h of culture was evaluated as described above.

Cell Stimulation and Lysis. For analysis of protein tyrosine phosphorylation and binding studies (see below), 2–3 × 10^7 thymocytes or 3 × 10^7 EL4 cells were resuspended in, respectively, 50 or 100 μl PBS, incubated for 30 min at 4°C in the presence or absence of 2.5 μg biotin-conjugated anti–mouse TCR antibody, and after the removal of unbound antibody, the cells were resuspended in 40 μl PBS and incubated at 37°C for varying periods of time with 100 μg/ml avidin. Alternatively, 3 × 10^7 thy-

mocytes or EL4 cells were resuspended in 100 μl PBS and then incubated for 2 min at 37°C with pervanadate (1 mM Na_3VO_4 plus 300 mM H_2O_2). After antibody or pervanadate stimulation, cells were pelleted by an 0.5-min centrifugation and then lysed by resuspension in 400 μl cold lysis buffer (150 mM NaC1, 50 mM Tris-HC1, pH 8, 2 mM EDTA, 50 mM NaF, 1 mM PMSF, 1 mM sodium orthovanadate, and 0.05% NaN₃) containing either 1% NP-40 or 0.5% Triton X-100 as detergents. After a 15-min incubation in lysis buffer, nuclei and unlysed cells were removed by centrifugation at 4°C for 10 min at 14,000 *g*, and 10^6 cell equivalents were then resuspended in SDS loading buffer and used as the source of protein lysates (see below).

Immunoprecipitation and Immunoblotting. Lysates were precleared before immunoprecipitation by incubating 1 mg lysate protein with protein A–Sepharose (Pharmacia, Baie d'Urfé, PQ) (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 specific antibody or rabbit preimmune serum and 25 μl packed protein A–Sepharose beads, and the immune complexes were then collected by centrifugation, washed four times in lysis buffer, and resuspended in SDS sample buffer. Alternatively, for reprecipitation studies, the immune complexes were collected by centrifugation, resuspended in 200 μl lysis buffer with 0.5% SDS, and then boiled for 5 min. The resolubilized proteins were then further diluted by resuspension in an additional 800 μl of lysis buffer and subjected to a second 3-h incubation at 4°C with 5 μl antibody and 25 μl protein A–Sepharose beads followed by centrifugation, washing in lysis butter, and resuspension is SDS. The samples were boiled for 5 min, electrophoresed through 10 or 15% SDS-polyacrylamide, transferred to nitrocellulose (Schleicher & Schuller Inc., Keene, NH), and incubated at 4°C for at least 1 h in TBST 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 primary antibodies (1/5,000 dilution) in TBST followed by the addition of goat anti–mouse antiserum labeled with peroxidase (Amersham) and horseradish peroxidase–conjugate (BioRad Laboratories, Hercules, CA). Immune complexes were detected using an enhanced chemiluminescence system (Amersham).

In Vitro Binding Assays. Protein binding to various GST–SH2 domain fusion proteins was examined by incubating total cell lysates (1 mg) for 1 h at 4°C with glutathione-Sepharose beads alone, and then for 3 h at 4°C with 5 μg glutathione–Sepharose-bound fusion protein. After four washes in lysis buffer, the precipitated complexes were resuspended in sample buffer, boiled, and analyzed by SDS-PAGE, and immunoblotted with selected antibodies as described above.

Assay of Phosphatase Activity. To measure PTP1C-induced dephosphorylation of Vav-associated proteins, Vav SH2–bound proteins prepared from 5 × 10^7 anti-TCR antibody–stimulated wild-type thymocytes were washed three times in lysis buffer and twice in phosphatase buffer (62 mM Hepes, pH 7, 6.25 mM EDTA), and then incubated at 37°C for varying periods of time in 25 μl phosphatase buffer containing 12.5 mM DTT and either 1 μg or no (control) GST full-length PTP1C fusion protein. Reactions were terminated by the addition of 6 μl 5× SDS loading buffer, and the samples were then subjected to immunoblotting analysis with antiphosphotyrosine antibody.

Assay of MAP Kinase Activity. Anti-ERK2 immunoprecipitates were prepared from 10^7 anti-TCR antibody– or PMA (25 nM)–stimulated wild-type thymocytes at varying intervals after cell stim-

ulation, and the immunoprecipitated proteins were washed and resuspended in 25 μl reaction buffer (30 mM Tris-HCl, pH 8.0, 20 mM Mg CI$_2$, 2 mM MnC1$_2$ containing 5 μg myelin basic protein (MBP; Upstate Biotechnology Inc.) and 10 μCi [γ^{32}-P] ATP (DuPont/New England Nuclear). After a 15-min incubation at 30°C, reactions were terminated by the addition of 6 μl 5× loading buffer, and the samples were then boiled, electrophoresed through 15% polyacrylamide gels, and transferred to nitrocellulose. The phosphorylated MBP bands were either visualized by phosphoimaging or stained with Pounceau S, excised from the filters, and γ^{32}-P incorporation was evaluated by liquid scintillation counting.

Results

TCR-elicited Proliferation Is Enhanced in me and me^v T Cells. Recent data implicating PTP1C in the modulation of numerous hemopoietic cell growth factor receptors and

indicating the substantial expression of this phosphatase in both thymocytes and mature peripheral T cells have revealed a likely role for PTP1C in the regulation of TCR signaling (15, 19–21). To begin elucidating PTP1C's influence on the ontogeny and activation of T cells, thymic cells isolated from PTP1C-deficient motheaten (*me*) and viable motheaten (*me^v*) mice were compared with normal thymocytes with respect to the surface expression of CD4, CD8, and TCR, as well as proliferative responses to TCR-dependent mitogenic stimuli. As shown in Fig. 1, results of three-color flow cytometry revealed no differences between *me^v* and control thymocytes with respect to the numbers of CD4, CD8, double-positive cells, and CD4 and CD8 single-positive cells and TCR expression. Thus, it appears that T cell differentiation can proceed normally in the absence of PTP1C, a conclusion consistent with previous data linking the progressive thymic involution that
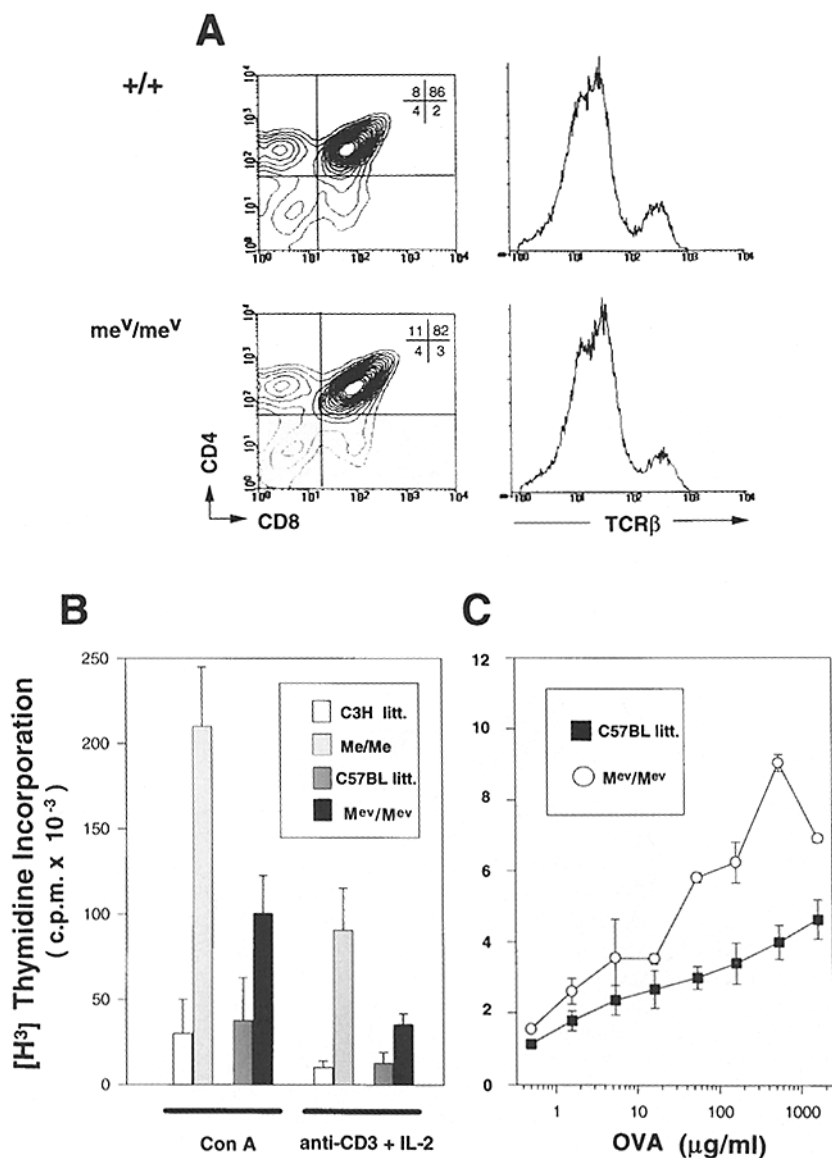

Figure 1. Comparison of T cell maturation and proliferative response to TCR stimulation in motheaten vs. wild-type mice. (*A*) Flow cytometric analysis of thymocytes from C57BL/6J +/+ (wild-type) and C57BL/6J *me^v/me^v* (Me^v) mice. Thymocytes isolated from 3-wk-old wild-type and mutant mice were stained with FITC-conjugated anti-CD4, PE-conjugated anti-CD8, and biotinylated anti-TCR-β antibodies followed by Tricolor-steptavidin. After staining, cells were analyzed by triple-color flow cytometry for the expression of CD4 vs. CD8 (*left panels*) and for levels of TCR expression as shown by the single parameter histograms on the right. For the two-color plots, the numbers in the upper right corners represent the percentage of cells found in each quadrant. (*B*) Proliferative responses of C57BL/6J *me^v/me^v* (Me^v) and normal littermates (*C57BL litt*) and C3 HeBFeJ *me/me* (Me) and normal littermates (*C3H litt*) to antigen receptor stimulation. Thymocytes (5 × 10^5 cells/well) were obtained from *me*, *me^v*, and congenic littermates, and were cultured for 48 h with 2.2 μg Con A in untreated microtiter plates or for 48 h in the presence of 25 U/ml IL-2 in microtitre plates precoated with anti-CD3 antibody. Proliferation was evaluated by measuring [^{3}H]thymidine incorporation during the last 6 h culture. Data are expressed as cpm, and the values represent means (± SEM) of quadruplicate cultures. (*C*) In vitro OVA-specific proliferative responses of C57 BL/6J *me^v/me^v* (Me^v) and congenic normal litter mate (*C57BL litt*) T cells after in vivo priming with OVA. Lymph nodes were harvested from *me^v* and normal littermate lymph nodes at 10 d after immunization with 50–100 μg OVA, as described in Materials and Methods. OVA-primed cells from two *me^v* mice or two congenic controls were pooled and mixed with equivalent numbers of lymph node cells derived from adjuvant-primed normal or *me^v* mice, respectively. Cells were cultured for 72 h at 5 × 10^5 cells/well in the presence of varying amounts of OVA, and proliferation was evaluated after a 6-h pulse with [^{3}H]thymidine. The values represent means (± SEM) of triplicate cultures.
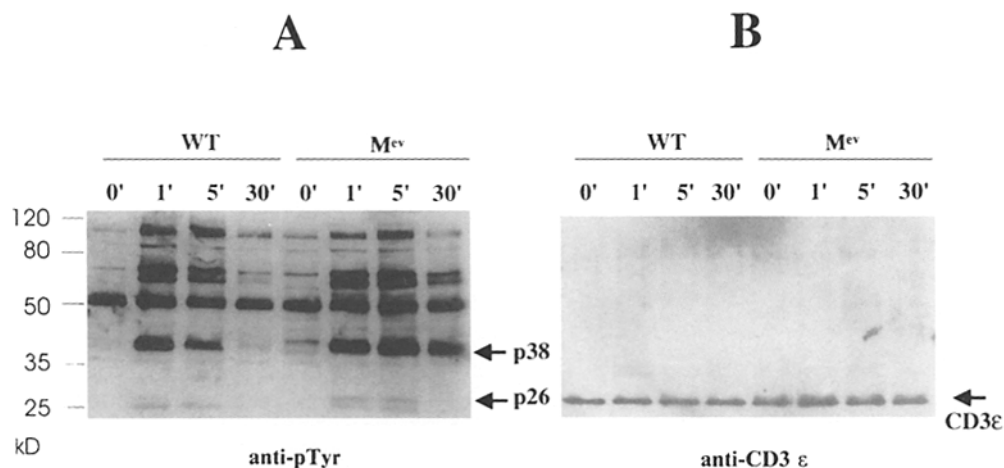
Figure 2. Comparison of TCR-induced protein tyrosine phosphorylation in viable moth-eaten vs. wild-type thymocytes. (*A*) Cell lysates were prepared from 10^6 unstimulated (O') or anti-TCR antibody (2.5 μg)/avidin–treated C57BL/6J *me^v/me^v* (Me^v) or C57BL/6J +/+ wild-type (*WT*) thymocytes at the indicated times after stimulation. After fractionation by SDS-PAGE, the phosphotryosine-containing proteins were detected by immunoblotting with antiphosphotyrosine antibody (*anti-pTyr*). The arrows indicate positions of two protein species (~38 and 26 kD, respectively) for which tyrosine phosphorylation appears enhanced in the *me^v* thymocytes. Mobilities of molecular mass standards are shown on the left. (*B*) The filter shown in *A* was stripped and reprobed with anti–CD3-ε antibody.

occurs in *me* and *me^v* mice primarily to the pathogenic effects of infiltrating myelomonocytic cells rather than to intrinsic T cell defects per se (25). In contrast to differentiation, however, thymocyte proliferative responses to TCR stimulation, as evaluated by [^{3}H]thymidine incorporation, differed markedly between the mutant mice and wild-type controls. *Me^v* thymocytes, for example, in which PTP1C catalytic activity is present, but at very low levels, exhibited maximal proliferative responses to both Con A and anti-CD3 antibody/IL-2 stimulation that were much greater in magnitude than those observed in similarly treated thymic cells from normal littermates (Fig. 1 *B*). Hyperresponsiveness to TCR stimulation was even more pronounced in thymocytes from *me* mice, which lack any PTP1C activity (Fig. 1 *B*), and was also revealed by the apparent capacity of both *me* and *me^v* thymic cells to proliferate in response to normally submitogenic concentrations of Con A (data not shown). By contrast, both the magnitude and kinetics of the proliferative responses to several TCR-independent stimuli, including IL-2 alone and PMA/ionomycin, were essentially the same in the mutant and wild-type thymocytes (data not shown). Together, these data reveal a defect in the transduction of TCR-evoked proliferation signals in *me* and *me^v* thymic cells and suggest that PTP1C effects on TCR-driven mitogenesis may be similar to those previously described in relation to the BCR (15).

To determine whether PTP1C also influences antigen receptor function in mature T cells, lymphocytes obtained from lymph nodes of OVA-primed *me^v* and wild-type congenic mice were compared with respect to their in vitro proliferative responses to OVA stimulation. Since these studies were performed using crude lymph node suspensions, the potential influence of contaminating antigen-presenting nonlymphoid cells was addressed by culturing the

OVA-primed *me^v* cells with CFA-primed cells from normal congenic mice in a 1:1 ratio. As shown in Fig. 1 *C*, the results of this analysis revealed the T cell proliferative responses to varying doses of antigen stimulation to be again substantially increased in *me^v* compared to control lymphocytes. While this result might reflect the capacity of a more permissive microenvironment to increase the sensitivity of the *me^v* cells to in vivo priming, this possibility seems unlikely in view of considerable data imbuing the *me* and *me^v* mutations with a predominantly inhibitory effect on lymphoid cell growth and function (26, 27). Instead, these data are more consistent with a role for PTP1C in regulating TCR-elicited mitogenesis in both mature T cells and thymocytes and suggest that the effects of PTP1C on TCR signaling are largely inhibitory.

TCR Complex Components Associate with PTP1C in Resting and Activated Thymocytes, and Are Hyperphosphorylated in the Context of PTP1C Deficiency. To elucidate the molecular basis for PTP1C influence on TCR-initiated T cell proliferation, *me^v* and normal thymocytes were next compared with respect to their profiles of protein tyrosine phosphorylation after TCR ligation. As demonstrated by antiphosphotyrosine immunoblotting analysis, anti-TCR antibody treatment resulted in the rapid tyrosine phosphorylation of a number of protein species in both the normal and *me^v* cells (Fig. 2 *A*). However, in contrast to the wild-type cells, in which tyrosine phosphorylation levels peaked ~1 min after TCR ligation, *me^v* thymocytes showed more sustained increases in tyrosine phosphorylation, with at least four phosphoproteins (75-, 70-, 38-, and 26-kD species) remaining relatively hyperphosphorylated at 5 and even 30 min after cell stimulation. Among these latter proteins, the phosphorylation of the 75- and 38-kD species also appeared relatively increased in unstimulated *me^v* compared to
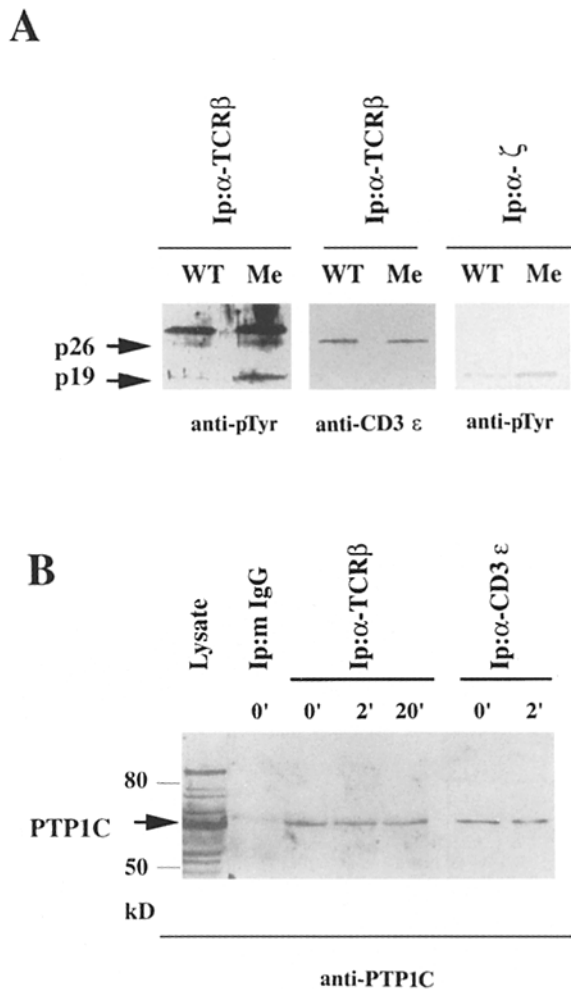
A

p26 →
p19 →

anti-pTyr   anti-CD3 ε   anti-pTyr

B

anti-PTP1C

Figure 3. Association of PTP1C with the resting and activated TCR complex. (*A*) Lysate proteins prepared from 3 × 10⁷ C3HeBFeJ *me/me* (Me) or C3HeBFeJ+/+ (*WT*) thymocytes were immunoprecipitated with either anti–TCR-β (*left and middle panels*) or anti-TCR-ζ (*right panel*) antibodies, resolved on SDS-PAGE, and subjected to immunoblotting with antiphosphotyrosine (*left and right panels*) or anti–CD3-ε (*middle panel*) antibodies. Arrows indicate the positions of two phosphoproteins showing relatively increased phosphorylation in the *me* compared to wild-type cells. (*B*) At the indicated times after stimulation with anti–TCR-β antibody/avidin, lysates prepared from 3 × 10⁷ EL4 cells or 3 × 10⁷ wild-type thymocytes (*last two lanes on the far right*) were immunoprecipitated with anti-TCR (Ip:TCR) or anti–CD3-ε (Ip:α-CD3ε) antibodies, or with an excess of purified murine IgG (Ip:mIgG) and the lysate (Lys) or immunoprecipitated proteins then resolved by SDS-PAGE and subjected to immunoblotting with anti-PTP1C antibody. An arrow on the left indicates the position of PTP1C. The positions of molecular mass markers are also shown on the left.

normal thymocytes (Fig. 2 *A*). These data, together with our detection of similar changes in tyrosine dephosphorylation kinetics in TCR-stimulated *me* thymocytes (data not shown), suggest that PTP1C effects on TCR-induced proliferation are realized, at least in part, at the earliest stages of signal transduction, and might include modulation of the TCR complex tyrosine phosphorylation status. To begin addressing this latter possibility, the immunoblot of wild-type and *me*ᵛ thymocyte proteins shown in Fig. 2 *A* was
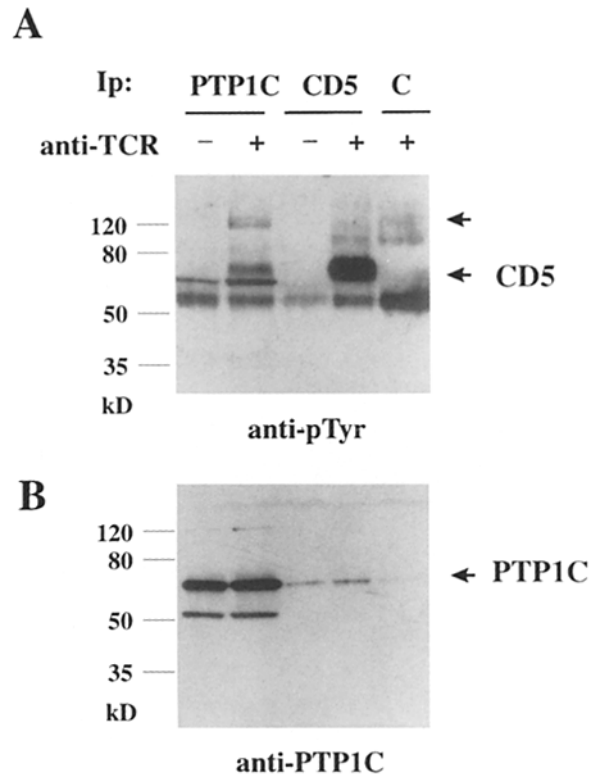
A

anti-pTyr

B

anti-PTP1C

Figure 4. Association of PTP1C with CD5 in resting and activated thymocytes. Lysates prepared from 3 × 10⁷ unstimulated (−) or anti-TCR antibody/avidin–stimulated (+) wild-type thymocytes were immunoprecipitated with anti-PTP1C or anti-CD5 antibodies or with rabbit preimmune serum (*C*), resolved over SDS-PAGE, and immunoblotted with antiphosphotyrosine (*A*) or anti-PTP1C (*B*) antibodies. The positions of CD5 and PTP1C are indicated on the right, and the positions of molecular mass standards are shown on the left.

reprobed with anti–CD3-ε antibody, and the comigration of this 26-kD TCR subunit with one of the proteins appearing hyperphosphorylated in TCR-stimulated *me*ᵛ thymocytes was thereby demonstrated (Fig. 2 *B*). Based on this observation, we next assessed the tyrosine phosphorylation status of TCR complexes immunoprecipitated from 0.5% NP-40 lysates of unstimulated wild-type and *me* thymocytes. As indicated by the antiphosphotyrosine immunoblot shown in Fig. 3 *A* (*left panel*), augmented tyrosine phosphorylation of several proteins in the size range of CD3 subunits (26 and 19 kD) was again detected in the *me* cells (Fig. 3 *A*), and the identity of these latter phosphoproteins as CD3-ε and TCR-ζ, respectively, was established by reprobing of the latter filter with anti–CD3-ε antibody, as well as by the demonstration of enhanced phosphorylation of TCR-ζ chains immunoprecipitated from *me* thymocytes (Fig. 3 *A*). Together, these data provide strong evidence for the hyperphosphorylation of the TCR components in PTP1C-deficient thymocytes, and thus identify the TCR subunits as potential substrates for PTP1C.

In view of these observations, the possibility that PTP1C physically associates with the TCR was also explored by anti-PTP1C immunoblotting analysis of TCR immuno-

precipitates from resting and anti-TCR antibody–treated EL4 thymoma cells. As shown in Fig. 3 *B*, PTP1C was easily detectable in the TCR precipitates derived from both resting and stimulated cells, the amount of TCR-associated PTP1C appearing essentially unchanged by cell stimulation. Similarly, PTP1C was coprecipitated with the TCR component CD3-ε, from both resting and stimulated thymocytes (Fig. 3 *B*), and phosphorylated species in the size range of TCR subunits were also detected by antiphosphotyrosine immunoblotting analysis of anti-PTP1C immunoprecipitates derived from wild-type thymocyte lysates (data not shown). Association of the TCR with PTP1C was also studied by examining interactions of this phosphatase with CD5, a membrane glycoprotein that associates with TCRζ and undergoes rapid tyrosine phosphorylation after TCR ligation consequent to interactions with src-related PTKs

(28–30). As shown in Fig. 4, the results of anti phosphotyrosine and anti-PTP1C immunoblotting analysis of CD5 immunoprecipitates from resting and anti-TCR–stimulated thymocytes confirmed that CD5 is rapidly phosphorylated after TCR ligation, and also demonstrated the capacity of PTP1C to associate with CD5 in activated thymocytes. In view of the recognized binding of CD5 molecules with the TCR complex, these observations provide further evidence for the physical association of PTP1C with the TCR.

Identification of Vav- and Grb2-associated Phosphoproteins as Substrates for PTP1C in Activated Thymocytes. In addition to interactions with various cell-surface receptors, PTP1C has recently been shown to asssociate with both the 95-kD product of the Vav protooncogene and the Grb2/mSos complex in activated hemopoietic cells (31). While the biochemical function of Vav is uncertain, Vav has been found
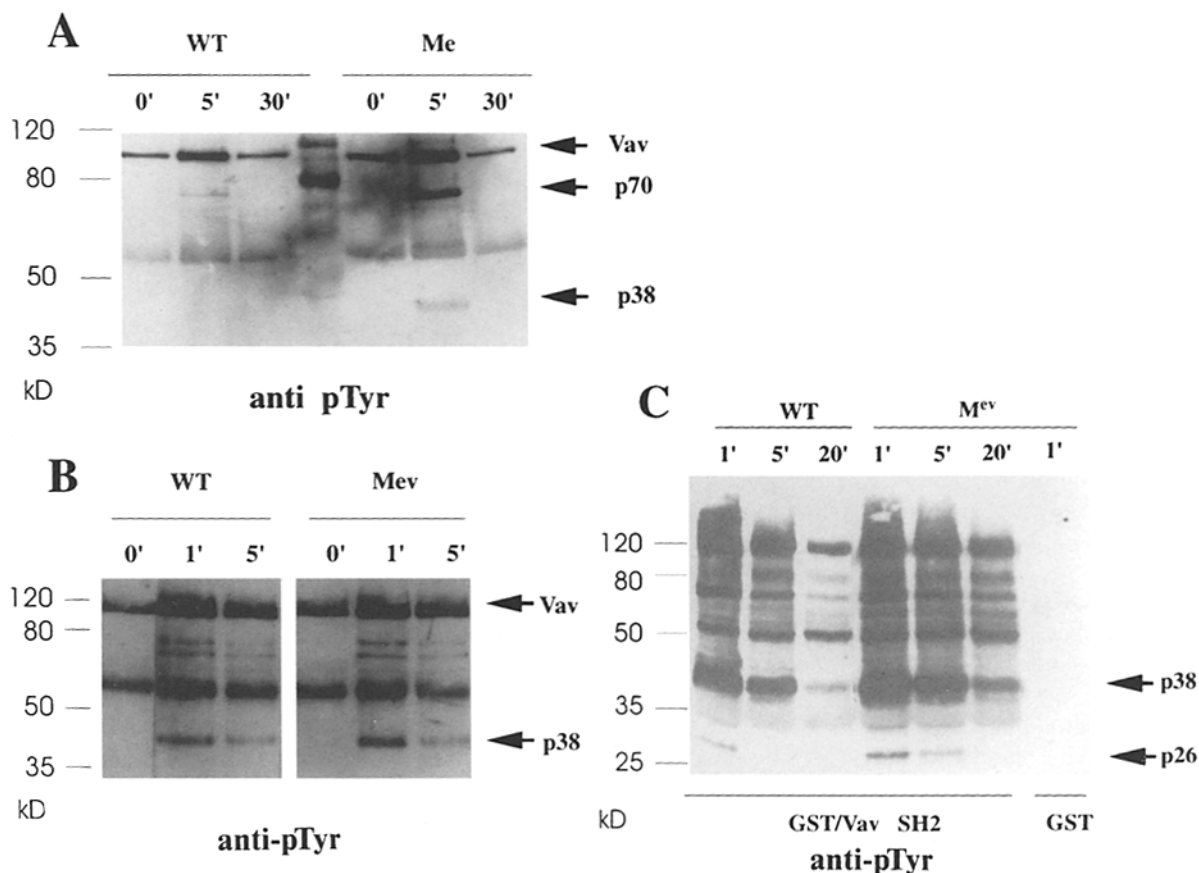

Figure 5. Phosphotyrosine protein binding to Vav is enhanced in PTP1C-deficient compared to normal activated thymocytes. (*A*) Lysates were prepared at the indicated times after Con A stimulation of 3×10^7 C3HeBFeJ *me/me* (Me) and congenic wild-type (*WT*) thymocytes. Lysate proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antiphosphotyrosine antibody. Arrows indicate the positions of Vav and two species showing increased tyrosine phosphorylation in the *me* compared to wild-type samples at 5 min after cell stimulation. (*B*) Lysates prepared at the indicated times after anti-TCR antibody/avidin stimulation of 3×10^7 C57BL/6J *me^v/me^v* (Me^v) and congenic, wild-type (*WT*) thymocytes were subjected to SDS-PAGE and antiphosphotyrosine immunoblotting analysis. Arrows indicate the positions of Vav and a 38-kD species showing increased phosphorylation in the *me^v* samples at 1 min after cell stimulation. (*C*) Lysates prepared from 3×10^7 C57BL/6J *me^v/me^v* (Me^v) and congenic wild-type (*WT*) thymocytes at the indicated times after anti-TCR antibody/avidin treatment were incubated for 1 h with 5 μg GST–Vav SH2 domain fusion proteins adsorbed to glutathione-Sepharose, and the bound proteins then fractionated by SDS-PAGE, transferred to nitrocellulose and subjected to immunoblotting with antiphosphotyrosine antibody. Arrows indicate the positions of two phosphoproteins (∼38 and 26 kD) that are more pronounced in the *me^v* relative to wild-type cells at 5 and 20 min after TCR stimulation. Precipitation with GST bound to glutathione-Sepharose (*far right lane*) was used as a negative control. For all panels, the positions of molecular mass markers are shown on the left side. In *A* and *B*, loading of equivalent amounts of lysate proteins was confirmed by reblotting with anti-Vav antibody (data not shown).

to have GEF activity for Ras in T lymphocytes (32), and has been identified as an essential participant in the cellular response to TCR stimulation by results showing that genetic ablation of Vav expression severely impedes TCR-evoked signal transduction and thymocyte differentiation (33, 34). Similarly, the Grb2 adaptor appears to be of considerable importance to T cell signaling by virtue of its role in coupling TCR-associated PTKs to the Ras GEF, mSos, and thus to the activation of Ras (35, 36). Accordingly, the capacity of PTP1C to associate with Vav and Grb2/mSos suggests that PTP1C influence on TCR signal transduction is also realized through its effects on downstream biochemical events that are required for activation of the Ras response pathway. To address this issue, the tyrosine phosphorylation of Vav and the association of Vav and Grb2 with other phosphotyrosine-containing proteins were examined in resting and antigen receptor–stimulated thymocytes of *me*, *me*ᵛ, and wild-type mice For these studies, lysates from Con A or anti-TCR antibody–treated mutant and control thymocytes were precipitated with anti-Vav or anti-Grb2 antibodies and glutathione–Sepharose-bound GST–Vav SH2 domain or GST–Vav Grb2 SH2 domain fusion proteins, and the precipitated complexes were then subjected to antiphosphotyrosine immunoblotting. As depicted in Figs. 5, *A* and *B*, the analysis of Vav immunoprecipitates in these cells showed that Vav is constitutively phosphorylated in both wild-type and mutant thymocytes, but, as has been previously reported, tyrosine phosphorylation of this protein increased rapidly after TCR ligation (23). This analysis also revealed the presence of several other phosphoproteins of ∼70-75 kD and of 38 kD, in the Vav immunoprecipitates from stimulated mutant and wild-type cells. However, in contrast to Vav, for which the levels and kinetics of tyrosine phosphorylation were comparable in the wild-type and mutant thymocytes, tyrosine phosphorylation of these latter Vav-associated phosphoproteins appeared, at least within the first 5 min of receptor stimulation, to be considerably more pronounced in *me* and to a lesser extent, *me*ᵛ thymocytes, relative to control thymocytes. This result cannot be ascribed to differences in the amount of Vav protein among the various samples, since reprobing of these immunoblots revealed the amount of Vav to be equivalent in all lanes (data not shown). Since increased and sustained tyrosine phosphorylation of proteins in this same approximate size range was also detected in whole-cell lysates from anti-TCR–stimulated *me*ᵛ thymocytes (Fig. 2), it appears likely that PTP1C modulates the tyrosine phosphorylation of these proteins and may thereby influence their association with Vav after TCR ligation. Conversely, while our previous data have revealed the association of Vav with both PTP1C protein and phosphatase activity (31), the current findings suggest that PTP1C does not modulate TCR-induced Vav tyrosine phosphorylation, and thus imply that any effects PTP1C exerts on the function of Vav in relation to T cell signal relay reflect the actions of this phosphatase on Vav-associated proteins and/or signaling effectors proximal to Vav activation.

Previous results from our group and others have demonstrated the capacity of both PTP1C and Vav to associate with the Grb2 adaptor protein, a molecule which by virtue of its SH2 and SH3 domains binds to both tyrosine phosphorylated receptor or receptor-associated adaptor proteins and to the mSos GEF, respectively, and thereby plays a key role in linking receptor to Ras activation in many cell types (36, 37). In view of these data, as well as the apparent influence of PTP1C on phosphoprotein binding to Vav after TCR ligation, the possibility that PTP1C modulation of receptor-triggered activation reflects its effects on tyrosine-phosphorylated signaling molecules normally recruited to the Vav and Grb2 SH2 domains was next examined. To this end, lysates from resting and stimulated control and *me*ᵛ thymocytes were incubated with GST-Vav SH2 domain and GST–Grb2 SH2 domain fusion proteins, and the SH2 domain–bound phosphoproteins were then visualized by antiphosphotyrosine immunoblotting analysis. As illustrated in Fig. 5 *C*, the results of this analysis revealed stimulated T cell lysates to contain multiple Vav SH2 domain–associated tyrosine phosphoproteins which, based on apparent molecular mass, include some of the same phosphorylated proteins (70–75 and 38 kD) coprecipitated with Vav from activated T cells (Fig. 5, *A* and *B*), as well as additional species of 120 and 26 kD, respectively. These findings are consistent with previous data that indicate association of the Vav SH2 domain with 70-kD and 74-kD phosphoproteins in activated Jurkat cells (38). Moreover, as the tyrosine phosphorylation of the Vav SH2–associated phosphoproteins detected in the stimulated thymocytes studied here was again more pronounced and sustained in *me*ᵛ compared to control cells, these results provide further evidence that PTP1C effects on T cell signal transduction involve the modulation of Vav SH2 domain–mediated interactions with phosphorylated signaling effectors, presumably because of PTP1C's capacity to tyrosine dephosphorylate at least some of these phosphoproteins.

Induction of tyrosine phosphoprotein binding to the Grb2 adaptor SH2 domain was also evaluated in anti-TCR–treated *me*ᵛ and wild-type thymocytes, and as shown in Fig. 6 *A*, this analysis revealed a prominent 38-kD phosphoprotein (herein designated as pp38) to be associated with Grb2 SH2 in both the *me*ᵛ and wild-type stimulated cells: again, the level and duration of pp38 tyrosine phosphorylation were augmented in *me*ᵛ relative to the control thymocytes. Similarly, a 38-kD phosphoprotein was the predominant molecular species coprecipitated with Grb2 from wild-type thymocytes after TCR ligation (Fig. 6 *B*). Since this species was found to comigrate with the Vav SH2 domain–associated 38-kD phosphoprotein and was no longer detectable in Grb2 SH2 domain precipitates derived from activated thymocyte lysates pretreated with an excess of GST–Vav SH2 domain fusion protein (data not shown), it appears likely that this 38-kD Grb2 SH2–binding phosphoprotein represents the same protein associated with the Vav SH2 domain in anti-TCR–stimulated thymocytes. While the identity of pp38 was not established here, a
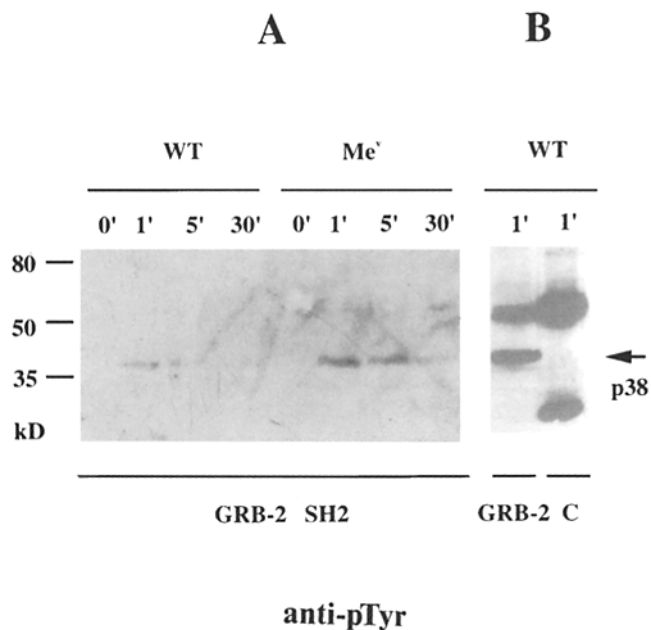
A   **B**

WT   Me⁺   WT

0' 1' 5' 30' 0' 1' 5' 30' 1' 1'

80 —

50 —

35 —

kD

p38

GRB-2 SH2   GRB-2 C

anti-pTyr

Figure 6. TCR-induced association of the Grb2 SH2 domain with p38 is increased in viable motheaten relative to normal thymocytes. (*A*) Lysates prepared from 3 × 10⁷ C57BL/6J *me*ᵛ/*me*ᵛ (Meᵛ) and congenic wild-type (*WT*) thymocytes at the indicated times after stimulation with anti-TCR antibody/avidin were incubated for 1 h with 5 μg GST-Grb2 SH2 domain fusion protein absorbed to glutathione-Sepharose. The precipitated phosphoproteins were then resolved by fractionation over SDS-PAGE and visualized by antiphosphotyrosine immunoblotting. Positions of molecular mass standards are shown on the left. (*B*) Lysate proteins from 3 × 10⁷ anti-TCR antibody/avidin-stimulated wild-type thymocytes were immunoprecipitated with anti-Grb2 antibody, resolved over SDS-PAGE, and immunoblotted with antiphosphotyrosine antibody. The arrow on the right indicates the position of p38.

phosphoprotein of a similar molecular mass (i.e., 36–38 kD) has been shown to interact with not only Grb2, but also PLC-γ1 and phosphatidylinositol-3 kinase in activated T cells (39–41). Based on these data, pp38 has been implicated in the coupling of TCR-associated PTKs to signaling effectors such as the Grb2/mSos complex, and accordingly to Ras activation (40), a hypothesis consistent with recent data linking the uncoupling of pp38 from the Grb2/mSos complex to the attenuation of the Ras activation response in T cells (42). While it is currently unclear whether the 38-kD species identified here represents Lnk, a recently isolated SH2 domain–containing protein with biochemical properties similar to those of pp38 (43), the current findings that reveal TCR ligation to induce pp38 association with not only the Grb2 SH2 domain, but also with the SH2 domain of Vav, a putative GDP/GTP exchanger for Ras, are consistent with the contended role for pp38 in the biochemical cascade linking TCR ligation to Ras nucleotide exchange. By extension, these data imply that PTP1C, by virtue of its effects on pp38 associations with both Vav and Grb2, plays a pivotal role in regulating receptor-initiated signaling events immediately upstream to Ras activation.

The enhanced tyrosine phosphorylation of various Vav- and Grb2-associated phosphoproteins detected in PTP1C-
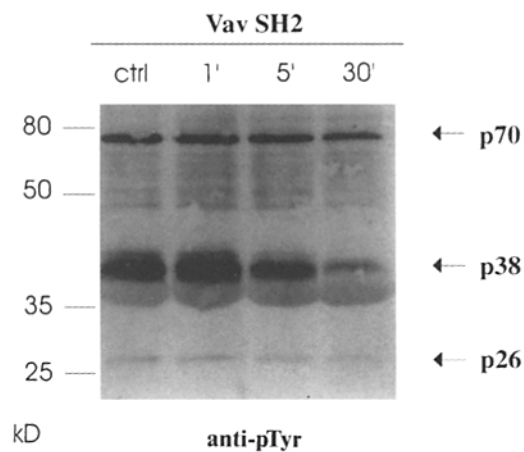

Vav SH2

ctrl   1'   5'   30'

80 —

50 —

35 —

25 —

kD

← p70

← p38

← p26

anti-pTyr

Figure 7. In vitro tyrosine dephosphorylation of Vav SH2-associated phosphoproteins by GST–PTP1C fusion protein. Lysates prepared from 5 × 10⁷ anti-TCR antibody/avidin–treated wild-type thymocytes were incubated for 1 h with 5 μg Vav SH2 domain fusion protein adsorbed to glutathione-Sepharose, and the complexes were then retrieved and incubated for the indicated times in phosphatase buffer alone (*ctrl*) or buffer containing 1 μg GST–PTP1C fusion protein. In vitro dephosphorylation was assessed by immunoblotting with anti–phosphotyrosine antibody. Arrows indicate positions of three prominent Vav SH2 domain–associated phosphoproteins of ∼70, 38, and 26 kD, respectively.

deficient thymocytes after TCR ligation suggested that these latter proteins represent targets for PTP1C-mediated dephosphorylation. To investigate this possibility, the capacity of PTP1C to tyrosine dephosphorylate Vav-associated phosphoproteins was examined by incubating GST–Vav SH2 domain precipitates from activated T cell lysates with recombinant PTP1C. As shown in Fig. 7, antiphosphotyrosine immunoblotting analyses of these PTP1C-treated precipitates revealed the progressive dephosphorylation of pp38, the latter being undetectable at 30 min after enzyme addition, as well as dephosphorylation of a second species of ∼26 kD, possibly representing a Vav-associated TCR component. These results do not appear to reflect the random targeting of all tyrosine phosphorylated species in the samples by the recombinant protein, since the phosphorylation state of a third Vav-binding phosphoprotein of 70 kD appeared to be unaffected by PTP1C, at least during the 30-min interval of this analysis. To further address the possibility that the 38- and 26-kD phosphoproteins represent PTP1C substrates, however, lysates prepared from wild-type thymocytes treated with pervanadate, a tyrosine phosphatase inhibitor known to induce intracellular changes that mimick those observed during TCR-triggered T cell activation (14), were precipitated with either GST–PTP1C or the catalytically inert GST–C/S PTP1C fusion proteins, and the effect of abrogating PTP1C activity on the enzyme's association with phosphoproteins in activated thymocytes was then assessed by antiphosphotyrosine immunoblotting. The results of this analysis revealed both versions of the PTP1C-GST fusion proteins to coprecipitate a number of phosphoproteins from stimulated T cells, but as shown in Fig. 8, many of these species appeared
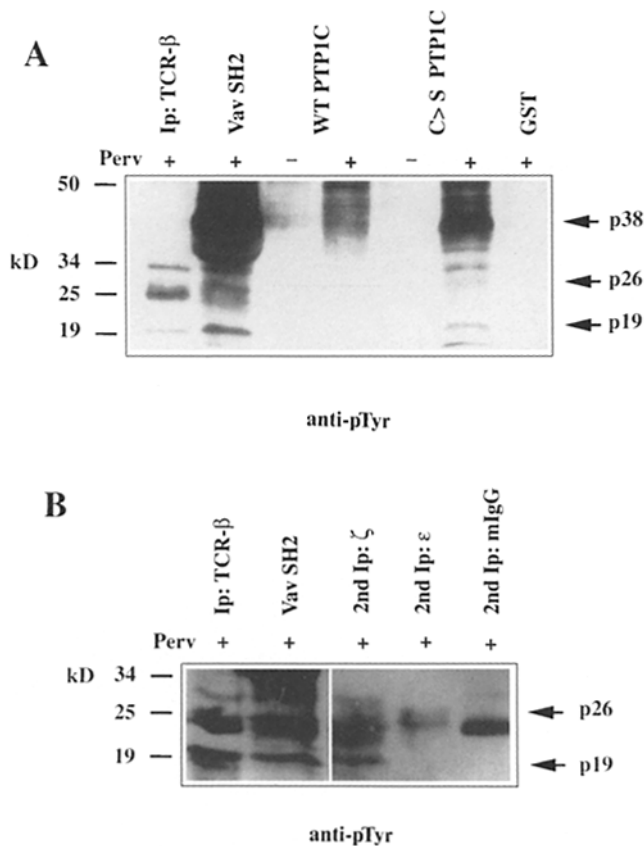
A

anti-pTyr

B

anti-pTyr

Figure 8. Association of PTP1C and the Vav SH2 domain with phosphorylated TCR components and p38. (*A*) Triton (0.5%) lysates prepared from 3 × 10⁷ unstimulated (−) or pervanadate-treated (+) wild-type thymocytes were precipitated with the indicated antibody (anti-TCR) or fusion protein (GST-C/S PTP1C, GST-PTP1C, or GST). The complexes were then fractionated over SDS-PAGE, transferred to nitrocellulose, and subjected to antiphosphotyrosine immunoblotting analysis. Arrows indicate the positions of comigrating tyrosine phosphorylated species detected in the GST-C/S PTP1C, Vav SH2, and anti-TCR precipitates. (*B*) For identification of the lower molecular mass phosphoproteins (19–26 kD) observed in *A*, 0.5% Triton lysates prepared from 3 × 10⁷ pervanadate-treated thymocytes were precipitated with either anti-TCR antibodies or GST–Vav SH2 domain fusion proteins and aliquots of the GST–Vav SH2 domain complexes were then reprecipitated with anti–TCR-ζ antibody, anti–CD3-ε antibody, or murine IgG, as described in Materials and Methods. The reprecipitated proteins were resolved over SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antiphosphotyrosine antibody. Arrows indicate the positions of the TCR-ζ (p19) and CD3-ε (p26) chains. In both panels, positions of molecular mass standards are shown on the left.

markedly more phosphorylated in the lysates precipitated with the catalytically inactive instead of the wild-type PTP1C fusion protein. Among these latter phosphoproteins, we detected species of 38, 26, 21, and 19 kD that comigrated with phosphoproteins precipitated from activated thymocyte lysates by anti-TCR antibodies and/or GST–Vav SH2 domain fusion proteins, and that are thus likely to represent the same molecular species found to be aberrantly phosphorylated in TCR and Vav immunoprecipitates from activated *me* and *meᵛ* thymocytes. This conten-

tion is supported by the results of reprecipitation analyses that were used to determine whether any of the 19–26-kD species precipitated from the activated cells by GST–Vav SH2 domain fusion proteins represented TCR components. As shown in Fig. 8 *B*, the results of this latter analysis revealed the presence of both TCR-ζ and CD3-ε in Vav SH2 domain precipitates from activated thymocytes. Thus, these data confirm the association of both Vav and PTP1C with TCR components in activated T cells and, based on the reduction in phosphorylation of the 19–26- and 38-kD proteins when precipitated with active vs. catalytically inert GST–PTP1C fusion proteins, these findings support the contention that pp38 and the phosphorylated components of the TCR complex represent substrates for PTP1C. While these observations might also be explained by indirect effects of PTP1C on the phosphorylation of these latter proteins, for example, through the modulation of one or more PTK activities, the capacity of PTP1C to associate with and tyrosine dephosphorylate in vitro at least some of these species (Fig. 7) strongly suggests that these phosphoproteins represent physiologic substrates for PTP1C. Accordingly, the negative regulatory effects of PTP1C on TCR signaling capacity appear to be realized through the dephosphorylation of activated TCR components, as well as the downstream signaling effectors that are normally involved in the induction of Ras activation.

TCR-evoked MAP Kinase Activation Is Prolonged in PTP1C-deficient Thymocytes. In view of these data implicating PTP1C in the regulation of the signaling cascades linking TCR stimulation to Ras activation, the influence of PTP1C on the Ras regulatory pathway was further examined by evaluating the effects of PTP1C deficiency on TCR-provoked activation of MAP kinase (MAPK). MAPKs belong to a group of serine/threonine kinases shown in many cell systems to engage in sequential phosphorylation cascades that direct Ras activation signals to the nucleus (44). With specific respect to antigen receptor signaling in T cells, the induction of MAPK activity appears tightly coupled to Ras activation, TCR-elicited increases in MAPK activity being essentially abrogated by the loss of Ras function and the genetic disruption of either Ras or MAPK function yielding very similar defects in thymocyte development in vivo (45, 46). Accordingly, to assess the influence of PTP1C on signaling events downstream to TCR-induced Ras activation, antibodies reactive with the ERK2 MAPK were used to immunoprecipitate MAPK from resting and anti-TCR stimulated *meᵛ* and wild-type thymocytes, and the immune complexes were then evaluated for their ability to phosphorylate MBP. As shown in Fig. 9 *A*, the results of this analysis indicate the rapid induction of increased MAPK activity in both the *meᵛ* and normal cells after TCR cross-linking. However, while the absence of PTP1C activity did not appear to influence basal levels of MAPK activity or the peak kinase activity observed immediately after receptor ligation (Fig. 9, *A* and *B*), the increases in MAPK activity induced by TCR stimulation were consistently prolonged in the *meᵛ* relative to the control cells. Thus, while MAPK ac-
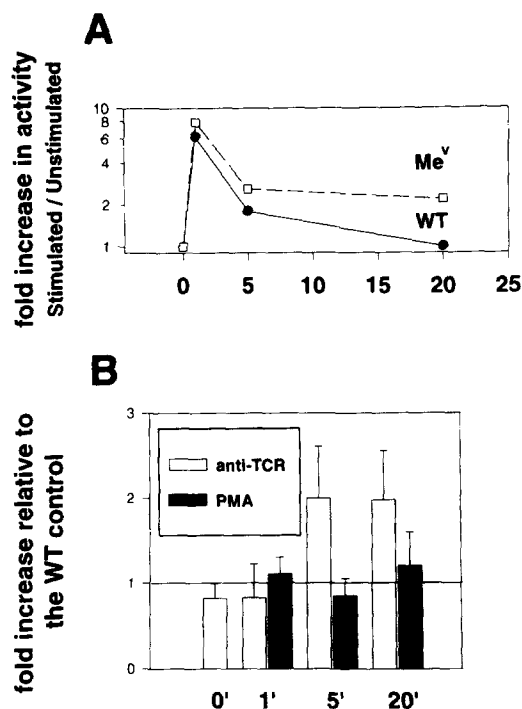
of Ras/MAPK, these findings identify PTP1C as a signaling element that is potentially involved in the attenuation of the Ras response to TCR ligation.

Discussion

The data reported in this paper indicate a significant role for PTP1C in regulating the T cell response to antigen receptor stimulation, and they suggest that PTP1C effects on the signaling pathways evoked by TCR engagement are predominantly inhibitory. The data link the negative influence of PTP1C on T cell signaling to this enzyme's interactions with and likely dephosphorylation of the resting and activated TCR complex components, as well as various cytosolic phosphoproteins, most notably pp38, which are recruited to the Vav and Grb2 SH2 domains after TCR engagement. The current findings strongly suggest that these latter phosphoproteins are directly dephosphorylated by PTP1C, but do not preclude the possibility that their phosphorylation state is modulated indirectly by PTP1C through its effects on PTK function, particularly in light of recent data implicating PTP1C in the negative regulation of Lck (19) and ZAP-70 (47) activities. While this latter issue remains to be resolved, the data reported here revealing the association of PTP1C deficiency with enhancement in TCR-induced cellular proliferation, activation of MAPK, and tyrosine phosphorylation of TCR components, as well as Grb2- and Vav-binding phosphoproteins, provide biochemical and functional evidence that PTP1C serves to downregulate the coupling of TCR stimulation to Ras activation and as such exerts a critical influence on the delivery of ligand-binding signals to the nucleus.

At the present time, the structural basis for PTP1C-TCR interaction is unclear, as this association does not appear to be substantially altered by receptor engagement and is therefore unlikely to be mediated through PTP1C SH2 domain binding to TCR phosphotyrosine residues. It is also unclear whether the increase in constitutive tyrosine phosphorylation of putative TCR elements detected in *me* thymocytes reflects ligand-independent receptor activation consequent to the lack of PTP1C activity, or alternatively, enhanced/prolonged stimulation of the TCR in vivo by ligands in the thymic environment. While these issues remain to be resolved, the data strongly suggest that PTP1C influence on TCR signaling is realized at least in part through the maintenance of the resting TCR in a tyrosine-dephosphorylated state, a role for PTP1C also previously proposed in relation to its effects on the BCR (15).

In addition to PTP1C association with TCR subunits, the current data also demonstrate the capacity of PTP1C to associate with CD5, a transmembrane protein expressed on both thymocytes and T cells and previously identified as a TCR-associated signaling element targeted for PTK-mediated tyrosine phosphorylation in activated T cells (30). In contrast to TCR-ζ and CD3-ε, CD5 association with PTP1C was somewhat enhanced in activated thymocytes in the context of CD5 tyrosine phosphorylation and may

Figure 9. Kinetics of MAPK activation are altered in PTP1C-deficient motheaten thymocytes. Lysates were prepared from 3 x 10⁷ anti-TCR antibody/avidin– or PMA-stimulated C57BL/6J *me*ᵛ/*me*ᵛ (Meᵛ) and congenic wild-type (*WT*) thymocytes at the indicated times after cell stimulation. Lysate proteins were then immunoprecipitated with anti-ERK2 antibody, and the immune complexes then evaluated for their ability to phosphorylate MBP after SDS-PAGE fractionation and autoradiography. (*A*) Representative example showing the kinetics of MAPK activation in *me*ᵛ-derived (*open squares*) and WT-derived (*closed circles*) thymocytes within the first 20 min after TCR stimulation. The incorporation of ³²P by the MBP substrate was quantitated by densitometric analysis (Molecular Dynamics PhosphorImager), and the values are expressed as the fold increase in MAPK activity (i.e., MBP phosphorylating activity in stimulated relative to unstimulated cells). (*B*) Comparison of *me*ᵛ vs. wild-type thymocytes with respect to levels of MAPK activity at varying times after TCR ligation or stimulation with 25 nM PMA. MBP phosphorylating activities of anti-ERK2 immunoprecipitates from the *me*ᵛ and wild-type anti-TCR–treated cells were again evaluated by SDS-PAGE and autoradiography, and the results were then quantitated by densitometric analysis of the phosphorylated bands. Results are expressed as the fold increase in MBP-phosphorylating activity of *me*ᵛ relative to wild-type–derived anti-ERK2 immunoprecipitates. The data shown represent the mean (± SEM) of values obtained in four independent experiments.

tivity fell rapidly to basal levels in the normal thymocytes, levels of this kinase activity remained markedly increased in the *me*ᵛ cells, even at 20 min after cell stimulation. By contrast, the magnitude and kinetics of MAPK activation after PMA stimulation were no different in the mutant and control cells (Fig. 9 *B*). Thus, the sustained increases in MAPK activity observed in the *me*ᵛ thymocytes after TCR stimulation cannot be ascribed to an intrinsic abnormality in MAPK function per se, but instead appear to reflect the consequence of defects in upstream molecular interactions through which Ras and MAPKs are recruited to the antigen receptor signaling pathway. Irrespective of the mechanisms whereby PTP1C deficiency perturbs the activation

therefore reflect the interaction of PTP1C SH2 domains with CD5 phosphotyrosine residues. While this possibility requires further analysis, the association of PTP1C with CD5 is of particular interest in view of recent data showing thymocyte hyperresponsiveness to TCR stimulation in mice rendered genetically deficient for CD5 expression (48). Since these latter data suggest that CD5 acts as a negative regulator of the TCR-mediated signal transduction events governing thymocyte selection, the capacity of PTP1C to associate with and potentially modulate CD5 tyrosine phosphorylation status raises the possibility that PTP1C inhibitory effects on TCR signaling are relevant to the regulation of intrathymic selection events. Although this hypothesis requires further investigation, a role for PTP1C in regulating TCR-mediated T cell selection is also implied by the current data suggesting that PTP1C acts to attenuate antigen receptor–triggered Ras/MAPK activation, a biochemical event recently shown to be required for TCR-induced positive selection (45, 46). Thus, while overt defects in thymocyte maturation were not detected in motheaten mice, the possibility remains that the inappropriate activation of Ras/MAPK pathways caused by PTP1C deficiency alters TCR signal circuitry in a manner that shifts the balance of positive and negative selection processes during thymocyte ontogeny. In other words, the effects of PTP1C deficiency on Ras activity may be associated with aberrant maturation of autoreactive thymocytes that are normally subject to intrathymic deletion. It is currently unknown whether the coupling of TCR-induced Ras activation to selection involves the upstream activation of CD5, but the suggested role for PTP1C in regulating TCR-driven thymocyte selection is consistent with the severe autoimmune syndrome expressed in motheaten mice and with previous data revealing an influence of this phosphatase on the signaling events coupling BCR engagement not only to cell proliferation/activation, but also to programmed cell death (16, 49). In addition, in view of the well-recognized link between aberrant Ras activation and malignant transformation, PTP1C effects on Ras may also be relevant to the transformation of lymphoid cells, a possibility supported by the increased susceptibility of *me* and *me* heterozygotes to lymphoproliferative disease (50).

Although the current data identify TCR components as well as pp38 and other Vav- and Grb2-binding phosphoproteins as likely substrates for PTP1C activity in activated thymocytes, the structural interactions that underlie these observations remain unclear. However, when considered in relation to previous data showing the capacity of PTP1C to interact with both Vav and Grb2 (31), as well as the current data demonstrating the binding of the Vav SH2 domain with phosphorylated components of the TCR, these findings are consistent with cumulative data in the literature linking intracellular signal transduction to the assembly of multimeric complexes within which various effectors interact so as to further propagate the activation signal. The likely juxtaposition of PTP1C, Grb2, and phosphorylated subunits of the TCR within this multimeric unit raises the possibility that PTP1C functions in this network in not only a regulatory capacity, i.e., through tyrosine dephosphorylation of other complex components, but also in an adaptor fashion, i.e., by providing a link between phosphorylated TCR subunits and the Grb2 SH2 domain. The posit of an adaptor role for PTP1C provides a molecular explanation for the mode of Grb2 recruitment into the signaling pathway originating from the TCR, and is also consistent with previous data revealing the structurally similar Syp tyrosine phosphatase to act as a linker for coupling-activated growth factor receptors to Grb2 (51). While a similar role is also subserved by the SH2 domain–containing Shc protein in relation to a number of growth factor receptors (52), Shc does not appear to play a predominant role in linking Grb2 to the activated TCR, since we (data not shown) and others (37) have not detected substantial levels of phosphorylated Shc in Grb2 or Grb2 SH2 domain precipitates from activated T cells. By contrast, in conjunction with published data showing Vav and Grb2 association to be mediated through dimerization of their respective SH3 domains (53), the current demonstration of Vav SH2 domain–mediated interaction with the phosphorylated TCR in activated thymocytes suggests that Vav function in T cell signaling may involve the coupling of Grb2 to the activated receptor components. In this context, the reported effects of Vav on Ras activity may derive from Vav-mediated enhancement of Grb2/mSos association rather than through Vav-induced GDP/GTP exchange on Ras, a possibility previously invoked to explain the discrepancy between Vav structural properties and putative biologic activity (53). In any case, irrespective of the extent to which Vav and/or PTP1C act as adaptors in relation to TCR signal relay, the data presented here suggest that the contributions of PTP1C and the signaling effectors with which it associates to the propagation of TCR-derived activation signals are likely to reflect the capacity of these molecules to participate in diverse combinations of molecular interactions and to thereby assume more than one function in relation to signal relay. Accordingly, the further exploitation of PTP1C-deficient mice toward the dissection of PTP1C functions in relation to the signaling transducing molecules with which it associates represents a promising avenue towards delineating the molecular mechanisms for translating TCR stimulation into a multiplicity of physiologic outcomes.

Address correspondence to Dr. Katherine Siminovitch, Mount Sinai Hospital, Room 656A, 600 University Avenue, Toronto, Ontario M5G 1X5, Canada.

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