

# Alterations in Activity of Protein Tyrosine Phosphatase SH-PTP1 in Autoimmune MRL/MpJ-lpr/lpr Mice<sup>1</sup>

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Activities of protein tyrosine kinase (PTK) and protein tyrosine phosphatase (PTP) in autoimmune MRL/MpJ-lpr/lpr mice (lpr mice) were measured and compared with the activities in the tissues from MRL/MpJ-+/+ mice (+/+ mice) as the control. In the spleen and liver, PTK activities in cytosol and membrane fractions were about 1.7- and 1.3-fold, respectively, higher in lpr mice than +/+ mice. PTP activities in cytosol and membrane fractions from lpr mice were 1.7- and 1.3-fold, respectively, higher in spleen, and 2.5- and 1.3-fold, respectively, higher in liver compared with those of the controls. These results demonstrate that the mutation of lpr gene resulted in elevation of PTK and PTP activities. Then, we measured the amounts and activities of SH-PTP1, a cytosolic PTP playing a crucial role in intracellular signaling from Fas antigen. The amounts of SH-PTP1 were about 4-fold larger in thymus, spleen, and lymphnodes than in liver, but there was no marked difference in the amounts between lpr and +/+ mice. On the other hand, activity of SH-PTP1 was definitely lower in lpr spleen and lymphnodes than +/+ spleen, but several times higher in lpr liver than +/+ liver. Tyrosine phosphorylation levels of SH-PTP1 in spleen of lpr and +/+ mice were similar. However, in liver, it was less phosphorylated in lpr than in +/+ mice. This hypophosphorylation might cause the activation of SH-PTP1 activity in lpr liver.

**Key words:** autoimmune disease, lpr mice, protein tyrosine phosphatase, SH-PTP1, tyrosine phosphorylation.

Protein tyrosine phosphorylation/dephosphorylation is a major regulatory mechanism in intracellular signaling associated with immune response, cell proliferation, and differentiation. In this study, we have determined both protein tyrosine kinase (PTK) and protein tyrosine phosphatase (PTP) activities in MRL/MpJ-lpr/lpr mice (lpr mice) in order to elucidate the roles and significance of protein tyrosine phosphorylation in autoimmune disease. Lpr mice develop a severe autoimmune disease that resembles systemic lupus erythematosus and rheumatoid arthritis in humans (1, 2). The predominant immunological feature in these mice is the development of peripheral lymphadenopathy due to the expansion of an abnormal T cell subset (TCR- $\alpha/\beta^+$ CD3<sup>+</sup>4<sup>-</sup>8<sup>-</sup>B220<sup>+</sup>) (3-5). Recently, it was demonstrated that the lymphoproliferation (lpr) is due to mutational defects of Fas antigen gene (6). Fas antigen is a cell-surface protein that mediates apoptosis.

PTK activation is an early and obligatory signaling event in Fas-induced apoptosis (7). On the other hand, it was recently reported that SH-PTP1-mediated protein dephosphorylation is also involved in the delivery of the Fas apoptosis signaling in lymphoid cells (8). SH-PTP1 (PTP1C, HCP, SHP) is one of the cytoplasmic PTPs, which is expressed predominantly in hematopoietic cells, and contains two tandemly aligned Src homology 2 (SH2) domains (9-12). In the mice homozygous for the recessive allelic mutation, motheaten or viable motheaten, point mutations have occurred at the SH-PTP1 gene which result in aberrant splicing of the SH-PTP1 transcript and cause severe immunodeficiency accompanied with systemic autoimmune disease (13-15). The disease reflects the presence of multiple hematopoietic cell abnormalities which include a marked overexpansion of the autoantibody-secreting CD5<sup>+</sup> B cells (16), impaired T cell and NK cell functions (17, 18), and increased production and tissue accumulation of granulocytes and monocyte/macrophages (19, 20). Both PTK and PTP are thus involved in regulation of protein tyrosine phosphorylation-mediated signaling pathways required for immune system response.

Here, we measured both PTK and PTP activities in lpr and +/+ mice, and then extensively investigated the amounts and activities of SH-PTP1 in lymphoid tissues from lpr mice and the controls. The significance of the results in relation to the autoimmune disease is discussed.

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Abbreviations: lpr mice, MRL/MpJ-lpr/lpr mice; NP-40, Nonidet P-40; pNPP, *p*-nitrophenyl phosphate; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; RCM-lysozyme, reduced carboxyamido-methylated and maleylated lysozyme; +/+ mice, MRL/MpJ-+/+ mice.

## MATERIALS AND METHODS

**Animals**—Male autoimmune-prone MRL/MpJ-lpr/lpr mice and MRL/MpJ-+/+ mice were purchased from Shizuoka Laboratory Animal Cooperative (Hamamatsu). All the mice used for experiments were aged 20 weeks.

**Tissue Extracts and Subcellular Fractionation**—Tissues were homogenized on ice with a hand homogenizer in 3 volumes of cold buffer containing 50 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 2 mM EDTA, 2 mM ethylene glycol-bis(2-aminoethylether)tetraacetic acid, 7 mM 2-mercaptoethanol, 0.1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 6 µg/ml antipain (buffer A). The homogenates were centrifuged at 8,000×*g* for 10 min at 4°C and the resulting supernatants were used as the crude extracts. The crude extracts were centrifuged at 105,000×*g* for 1 h at 4°C and the resulting supernatants were used as the cytosol fractions. The precipitates were rinsed 5 times with buffer A and extracted with buffer A containing 0.5% (w/v) Nonidet P-40 (NP-40) (Sigma Chemical, St. Louis, MO). After centrifugation at 105,000×*g* for 1 h at 4°C, the resulting supernatants were used as the membrane fractions.

**Tyrosine Kinase Assay**—Tyrosine kinase activity was determined by measuring the amount of <sup>32</sup>P incorporated into tyrosine-alanine-glutamate copolymers. The standard assay mixture (50 µl) contained 10–25 µg protein, 25 mM Hepes, pH 7.5, 25 mM MgCl<sub>2</sub>, 0.1 mM sodium vanadate, 0.2 mM ATP (Sigma Chemical), [ $\gamma$ -<sup>32</sup>P]ATP (100–400 cpm/pmol, Amersham International plc, England), 10% (v/v) glycerol, 0.5% NP-40, and 0.4 mg/ml poly (Glu : Ala : Tyr; 6 : 3 : 1) (Sigma Chemical). After incubation for 20 min at 30°C, the reaction mixture (40 µl) was spotted on a square (2×2 cm) of phosphocellulose paper (P81, Whatman, England), which was then washed 3 times with 75 mM phosphoric acid and dried. The radioactivity was measured with a liquid scintillation counter. One unit (U) of PTK activity was defined as the amount of enzyme that catalyzes the incorporation of 1 nmol of phosphate per min.

**Tyrosine Phosphatase Assay**—Tyrosine phosphatase activity was measured in terms of the release of radiolabeled phosphate from the reduced carboxyamido-methylated and maleylated lysozyme (RCM-lysozyme) as described (21–24). RCM-lysozyme was prepared by the procedure of Tonks *et al.* (23). <sup>32</sup>P-RCM-lysozyme was prepared by incubating RCM-lysozyme with rabbit spleen protein tyrosine kinase in a reaction mixture of 25 mM Hepes, pH 7.2, 25 mM MgCl<sub>2</sub>, 0.1 mM sodium vanadate, and 0.4 mM [ $\gamma$ -<sup>32</sup>P]ATP (400 cpm/pmol) for 6 h at 30°C. The lysozyme was exclusively phosphorylated on tyrosine (>99%) and the phosphorylation rate was 0.3 mol/mol protein. The tyrosine kinase used in this reaction was purified from rabbit spleen as described (21, 22). The reaction mixture for PTP assay contained 25 mM imidazole-HCl, pH 7.2, 1 mg/ml BSA, 0.5% NP-40, 280 mM 2-mercaptoethanol, 30 µM <sup>32</sup>P-RCM-lysozyme, and tissue extract in a final volume of 60 µl. After 10 min at 30°C, the reaction was terminated with an equal volume of 20 mM silicotungstic acid in 0.01 M H<sub>2</sub>SO<sub>4</sub>. After 10 min on ice, the mixture was centrifuged at 12,500×*g* for 2 min. The supernatant was mixed with one-fifth volume of 5% ammonium molybdate in 2 M H<sub>2</sub>SO<sub>4</sub> and extracted with an equal volume of isobutanol/

benzene (1 : 1). The radioactivity in the upper phase was measured with a liquid scintillation counter. One unit (U) of PTP activity is defined as the enzyme activity that releases 1 nmol of phosphate per min.

**Western Blot Analysis**—Western blot analysis was carried out essentially as described by Takizawa *et al.* (25). The membrane was blocked with phosphate-buffered saline containing 3% bovine serum albumin (fatty acid-free, Sigma Chemical) and 0.1% (v/v) Tween-20 and incubated with 1 µg/ml anti-SH-PTP1 polyclonal antibody (UBI, Lake Placid, NY) and then 1 µg/ml horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody (Chemicon, Temecula, CA), or with 1 µg/ml anti-phosphotyrosine monoclonal antibody (PY-20) (ICN, Costa Mesa, CA), and then 1 µg/ml horseradish peroxidase-conjugated goat anti-mouse IgG ( $\gamma$ ) antibody (KPL, Gaithersburg, MD). Immunoreactive bands were detected with an ECL Western blotting detection kit (Amersham International plc).

**Immunoprecipitation and Measurement of SH-PTP1 Activity**—Immunoprecipitation of SH-PTP1 was carried out essentially by the method of Li *et al.* (26) with a slight modification. The crude tissue extracts containing up to 500 µg protein were diluted to 2.5 mg/ml with cold RIPA buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 2 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride), and mixed by inversion for 30 min at 4°C. The diluted extracts were precleared with 10 µl of Protein A-Sepharose 4FF (Pharmacia, Uppsala, Sweden) for 1 h at 4°C. After brief centrifugation, supernatants were incubated with 5 µg/ml anti-SH-PTP1 antibody for 2 h at 4°C followed by incubation with 10 µl of Protein A-Sepharose 4FF for 1 h at 4°C. The Sepharose beads were then washed once with washing buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% NP-40), twice with WG buffer (50 mM Hepes, pH 7.6, 150 mM NaCl, 0.1% NP-40), and twice with assay buffer (100 mM sodium acetate, pH 5.0, 1.6 mM dithiothreitol). The beads were incubated with 200 µl of the assay buffer containing 10 mM *p*-nitrophenyl phosphate (pNPP) at 30°C for 1 h under shaking. The reaction was terminated by adding 1 ml of 0.2 M NaOH and the absorbance at 410 nm was determined.

**Protein Determination**—Protein was measured with DC Protein Assay (Bio-Rad, Hercules, CA), using bovine serum albumin as the standard.

## RESULTS

**Activities of PTK and PTP in lpr and Control Mice**—In our preliminary experiments on Western blot analysis using anti-phosphotyrosine antibody, we found various alterations in protein tyrosine phosphorylation in extracts from lpr lymphoid tissues (data not shown). These results suggested that PTK and/or PTP might be altered in mice with this autoimmune disease. To investigate the regulatory mechanism(s) of alterations in protein tyrosine phosphorylation, we first measured PTK activities in lpr and control mice by using poly (Glu : Ala : Tyr; 6 : 3 : 1) as the substrate. Table I shows PTK activities in cytosol and membrane fractions. In both lpr and control mice, the activities in spleen were much higher than those in liver, and the activities in membrane fraction were higher than those in cytosol fraction. In both spleen and liver, PTK

activities in *lpr* mice were elevated about 1.7-fold in cytosol fraction and 1.3-fold in membrane fraction compared with those of the controls.

PTP activities were then measured by using  $^{32}\text{P}$ -tyrosine-labeled RCM-lysozyme as the substrate. PTP in membrane fraction is totally dependent on 0.5% NP-40, whereas PTP in cytosol fraction does not require it for activity (data not shown). As shown in Table I, PTP activities in cytosol fractions of spleen and liver were elevated about 1.7- and 2.5-fold, respectively, in *lpr* mice compared with those of the controls. PTP activities in membrane fraction of both spleen and liver were only 1.3-fold higher in *lpr* than the controls. These results demonstrate that both PTK and PTP activities are elevated in *lpr* mice.

Since SH-PTP1 is thought to play a crucial role in intracellular signaling from Fas antigen, the contribution of SH-PTP1 to the increase of PTP in *lpr* mice was then evaluated by using a specific polyclonal antibody against SH-PTP1. As shown in Fig. 1, upon preincubation of extracts with the anti-SH-PTP1 antibody, PTP activities in liver of both *lpr* and *+/+* mice were decreased, but the

TABLE I. PTK and PTP activities of the spleen and liver of *+/+* and *lpr* mice. PTK activities of the spleen and liver of *+/+* and *lpr* mice were measured by using 0.4 mg/ml poly (Glu : Ala : Tyr; 6 : 3 : 1) as the substrate. PTP activities were measured by using  $^{32}\text{P}$ -RCM-lysozyme as the substrate. The data represent the means  $\pm$  standard deviation of three separate experiments, in units/mg protein.

|        |            | PTK             |                 | PTP            |                |
|--------|------------|-----------------|-----------------|----------------|----------------|
|        |            | Cytosol         | Membrane        | Cytosol        | Membrane       |
| Spleen | <i>+/+</i> | 0.20 $\pm$ 0.01 | 0.87 $\pm$ 0.08 | 10.2 $\pm$ 0.8 | 35.2 $\pm$ 2.5 |
|        | <i>lpr</i> | 0.34 $\pm$ 0.08 | 1.15 $\pm$ 0.22 | 17.1 $\pm$ 2.6 | 44.3 $\pm$ 2.4 |
| Liver  | <i>+/+</i> | 0.02 $\pm$ 0.01 | 0.20 $\pm$ 0.02 | 1.6 $\pm$ 0.5  | 4.0 $\pm$ 0.8  |
|        | <i>lpr</i> | 0.03 $\pm$ 0.02 | 0.29 $\pm$ 0.05 | 3.9 $\pm$ 0.4  | 5.3 $\pm$ 0.2  |

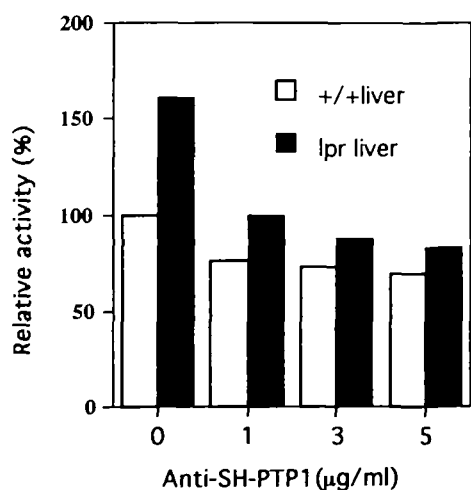


Fig. 1. Amount of SH-PTP1 activity in the total PTP activity of liver extract. Liver crude extracts from *+/+* (open bar) and *lpr* (closed bar) mice containing 200  $\mu\text{g}$  protein were diluted to 1 mg/ml protein with RIPA buffer, and immunoprecipitated with the indicated concentrations of anti-SH-PTP1 antibody and 10  $\mu\text{l}$  of protein A-Sepharose 4FF. PTP activities of the resulting supernatants after 1,000-fold dilution were measured with  $^{32}\text{P}$ -RCM-lysozyme. All data points are means of duplicate determinations.

extent of the decrease was much greater in *lpr* than in *+/+* mice. These results strongly suggest that the increase in PTP activity in *lpr* liver is mostly attributable to the increase of SH-PTP1 activity. We then investigated the alterations of SH-PTP1 in *lpr* mice.

*Up- and Down-Regulation of SH-PTP1 Activity in Liver and Spleen, Respectively, in lpr Mice*—SH-PTP1 activity was measured by using the immunoprecipitates formed with anti-SH-PTP1 antibody as the enzyme and pNPP as the substrate under conditions where the activity increased linearly with increasing amounts of the extract and was completely inhibited by 1 mM vanadate (data not shown). Figure 2C shows SH-PTP1 activities in lymphoid tissues from *lpr* and *+/+* mice. In *+/+* mice, SH-PTP1 activity was much higher in thymus and spleen than in liver (lanes 1, 2, and 4). It should be noted that SH-PTP1 activity in *lpr* spleen was slightly but significantly lower than that of *+/+* spleen (lanes 2 and 3), although the total PTP activity measured with RCM-lysozyme as the substrate was increased, as described above (Table I). These results suggest that alteration patterns of PTPs in *lpr* mice are isoform-dependent. The SH-PTP1 activity in *lpr* lymphnodes was similar to that in *lpr* spleen (lanes 3 and 6). It should also be

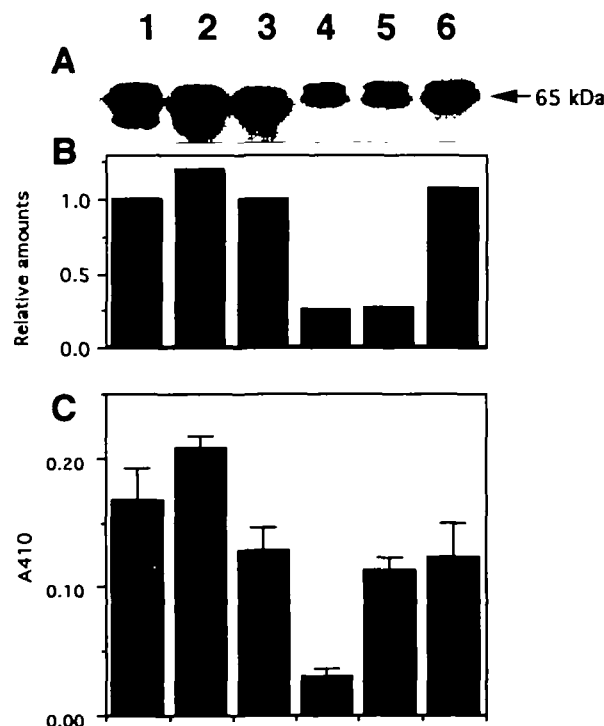
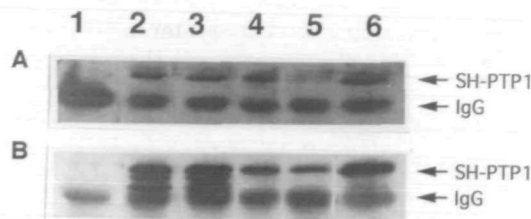


Fig. 2. Expression and activity levels of SH-PTP1 in *+/+* and *lpr* mice. (A) Western blot analysis. The crude extracts containing 10  $\mu\text{g}$  protein from *+/+* and *lpr* mice were subjected to Western blot analysis with anti-SH-PTP1 antibody. Lanes: 1, *+/+* thymus; 2, *+/+* spleen; 3, *lpr* spleen; 4, *+/+* liver; 5, *lpr* liver; and 6, *lpr* lymphnodes. (B) The intensity of the bands was evaluated with a scanning densitometer. (C) Activity of SH-PTP1. The crude extracts containing 500  $\mu\text{g}$  protein were immunoprecipitated with anti-SH-PTP1 antibody. The pNPP activity of the precipitates was assayed as described under "MATERIALS AND METHODS." The value of the absorbance observed without the antibody was subtracted from each value. The data represent the means  $\pm$  standard deviation of 4 separate experiments.



**Fig. 3. Tyrosine phosphorylation levels of SH-PTP1 in +/+ and *lpr* mice.** Tissues were extracted with buffer A containing 1 mM sodium vanadate and 50 mM NaF. The extracts containing 200  $\mu$ g protein were diluted to 1 mg/ml protein with RIPA buffer containing 1 mM sodium vanadate and 50 mM NaF, and subjected to immunoprecipitation with anti-SH-PTP1 antibody and protein A-Sepharose 4FF. Lanes 1, pre-clean; 2, +/+ spleen; 3, *lpr* spleen; 4, +/+ liver; 5, *lpr* liver; and 6, *lpr* lymphnodes. The Sepharose beads were washed 5 times with RIPA buffer containing 1 mM sodium vanadate and 50 mM NaF, and eluted with the sample buffer of SDS-PAGE. The eluates were analyzed by Western blot analysis with anti-phosphotyrosine antibody (A). The membrane was then incubated with 63 mM Tris-HCl, pH 6.7, 2% SDS, 0.1 M 2-mercaptoethanol for 1 h at 60°C to remove the antibodies. It was reprobed with anti-SH-PTP1 antibody (B). Three independent experiments gave similar results.

noted that SH-PTP1 activity in liver was markedly increased in *lpr* mice as compared with the control (lanes 4 and 5).

**Regulation Mechanism of SH-PTP1 Activity**—Figure 2 (A and B) shows the results of Western blot analysis of SH-PTP1. The amounts of SH-PTP1 in lymphoid tissues including thymus and spleen were at similar levels, and were 4–5 times higher than those of liver. The amounts in liver and spleen of *lpr* mice were not different from those in control mice, although the SH-PTP1 activity was up- and down-regulated in these tissues, respectively, in *lpr* mice (Fig. 2C).

SH-PTP1 possesses several tyrosine phosphorylation sites which are phosphorylated by PTKs in response to hormones or cytokines. Therefore, to elucidate the relationship between amount and activity of SH-PTP1, crude extracts from *lpr* and control tissues were immunoprecipitated with anti-SH-PTP1 antibody, and the precipitates were subjected to Western blot analysis with anti-phosphotyrosine antibody (PY-20). As shown in Fig. 3A, tyrosine phosphorylation levels of SH-PTP1 in spleen of *lpr* and +/+ mice were similar (lanes 2 and 3), whereas that in liver was definitely lower in *lpr* than in +/+ mice (lanes 4 and 5). On reprobing of the same membrane with anti-SH-PTP1 antibody, the amounts of SH-PTP1 in liver of +/+ and *lpr* mice were almost the same (Fig. 3B, lanes 4 and 5).

#### DISCUSSION

Our results may be summarized as follows. (i) Both PTK and PTP activities were increased in *lpr* mice. (ii) Although total activity of PTP measured with RCM-lysozyme as the substrate was increased in *lpr*, SH-PTP1 activity was decreased in spleen of *lpr* mice to the level in *lpr* lymphnodes, about 70% of that in +/+ spleen. In contrast, SH-PTP1 activity in liver was elevated several-fold in *lpr* mice compared with that in +/+. (iii) SH-PTP1 in *lpr* liver was hypophosphorylated at the tyrosine residue(s) compared with that in +/+ liver.

Increases in both PTK and PTP activities in *lpr* mice may induce (i) acceleration of turnover of protein tyrosine phosphorylation in *lpr* mice, and/or (ii) variations of tyrosine phosphorylation pattern of proteins in *lpr* mice due to different substrate specificity. Our preliminary results of Western blot analysis demonstrate that the tyrosine phosphorylation levels were not different in most proteins between +/+ and *lpr* mice, but those of some proteins were significantly different (data not shown).

Although SH-PTP1 activity was significantly lower in *lpr* spleen than in +/+ spleen, the decrease was very small compared with that in motheaten mice, which have another autoimmune disease. SH-PTP1 is thought to be located downstream from Fas-antigen in Fas-mediated apoptosis signaling (8). In motheaten mice carrying loss-of-function mutation in SH-PTP1 gene, this functional defect causes severe autoimmune disease (13–15). However, their Fas gene remains intact. In *lpr* mice, on the other hand, mutation occurred on the Fas gene, but the SH-PTP1 gene is intact. The decrease in SH-PTP1 activity in *lpr* lymphoid tissues was only 30%, suggesting that the Fas-mediated signaling remains intact downstream of Fas antigen.

It should be noted that SH-PTP1 in *lpr* liver is hypophosphorylated at tyrosine residue(s). The hypophosphorylation may cause activation of its PTP activity, at least in liver. The remarkable increase in SH-PTP1 activity in *lpr* liver has two possible causes: (i) the decrease in tyrosine phosphorylation levels and (ii) accumulation of *lpr* double-negative lymphocytes in liver. In *lpr* liver, the amount of SH-PTP1 was not increased (Fig. 2A). Therefore, the increase of SH-PTP1 activity in *lpr* liver may be mainly due to the decrease in tyrosine phosphorylation levels, or activation of SH-PTP1 by another modification may induce auto-dephosphorylation in *lpr* liver. Several lines of evidence indicate that SH-PTP1 is phosphorylated on its tyrosine residues in response to extracellular stimulations. Uchida *et al.* reported activation of SH-PTP1 through tyrosine phosphorylation by insulin receptor kinase (27). However, SH-PTP1 phosphorylated on C-terminal tyrosyl residues by stimulation of CD4 or CD8 did not change its activity (28). In any case, these results suggest that SH-PTP1 is functionally regulated by its reversible tyrosine phosphorylation. It was also reported that SH-PTP1 contains possible serine phosphorylation sites for protein kinase C (29) and mitogen-activated protein kinase (30, 31). So far, there is no direct evidence indicating that hypophosphorylation of SH-PTP1 causes its activation. But, the above results raise the possibility that hypophosphorylation on tyrosine residue(s) of SH-PTP1 in *lpr* liver occurred by increased auto-dephosphorylation activity after reversible phosphorylation on serine/threonine residues. We previously reported specific increases in the activities of PP2A and PP2B, serine/threonine protein phosphatases, in *lpr* mice (32–34). Taken together, our present results on PTK, PTP, and SH-PTP1 in *lpr* mice provide further insight into the pathogenesis of an autoimmune disease.

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