A Cytosolic Protein-tyrosine Phosphatase PTP1B Specifically Dephosphorylates and Deactivates Prolactin-activated STAT5a and STAT5b*

Naohito Aoki‡ and Tsukasa Matsuda

From the Department of Applied Molecular Biosciences, Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan

Prolactin (PRL) plays a central and crucial role in the regulation of milk protein gene expression in mammary epithelial cells. PRL binding to its cognate receptor leads to receptor dimerization and activation of the tyrosine kinase Janus kinase 2 (JAK2), associated with the membrane-proximal, intracellular domain of the receptor. In turn, JAK2 phosphorylates and activates STAT5, a member of the signal transducers and activators of transcription (STAT) family. We have recently reported that 16 different protein-tyrosine phosphatases (PTPs) were expressed in lactating mouse mammary gland and mammary epithelial cells, and that 16 different protein-tyrosine phosphatases (PTPs) were expressed in lactating mouse mammary gland and mammary epithelial cells (Aoki, N., Kawamura, M., Yamaguchi-Aoki, Y., Ohira, S., and Matsuda, T. (1999) J. Biochem. (Tokyo) 125, 669–675). We investigated the involvement of each PTP in PRL signaling. Among the 16 phosphatases including SHP-2 examined, a cytosolic phosphatase PTP1B was found to specifically dephosphorylate STAT5a and STAT5b in transfected COS7 cells in vitro. Nuclear translocation of STAT5a and STAT5b was largely inhibited upon overexpression of PTP1B.

The PRL-dependent transcription of the β-casein gene promoter was also inhibited by PTP1B. Furthermore, retrovirus-mediated overexpression of PTP1B resulted in down-regulation of STAT5 and down-regulation of the expression of β-casein in mammary epithelial COMMA-1D cells. Concanavalin A was tyrosine-phosphorylated STAT5a and STAT5b expressed in COS7 cells were co-precipitated by substrate-trapping mutants of recombinant PTP1B. These results strongly suggest that PTP1B dephosphorylates PRL-activated STAT5a and STAT5b, thereby negatively regulating PRL-mediated signaling pathway. The polypeptide hormone prolactin is produced in the anterior pituitary, regulates the activity of milk protein gene promoters in mammary epithelial cells, and plays an important role in the growth and differentiation of lymphocytes (1). It exhibits its activity via its cognate receptor and the activation of intracellular signaling molecules such as the Janus kinase (JAK) signal transducers and activators of transcription (STAT) pathway. The prolactin (PRL) receptor, belonging to the hematopoietin receptor superfamily (2), does not possess intrinsic tyrosine kinase activity but is associated with the cytoplasmic tyrosine kinase JAK2 (3–5). Ligand binding leads to dimerization of the receptor and activation of JAK2 (5). JAK2 phosphorylates not only the prolactin receptor but also the transcription factor STAT5. Upon phosphorylation, STAT5 forms homodimers, translocates to the nucleus, and specifically binds to the promoter of target genes, thus activating transcription.

Two closely related STAT5a and STAT5b have been identified. They are very similar but different (6, 7). They have 93% identity at the amino acid level and are found in all hematopoietic and non-hematopoietic cells. STAT5a is ubiquitously expressed in most cell lines and tissues at comparable levels with a few exceptions (10). STAT5a and STAT5b are also activated by other cytokines, including growth hormone, erythropoietin, granulocyte macrophage-colony stimulating factor (14, 15), thrombopoietin (16), interleukin (IL)-2 (17, 18), IL-3 (8, 14), IL-5 (14), IL-7, IL-15 (18), as well as epidermal growth factor through its respective receptor tyrosine kinase and by non-receptor tyrosine kinases Src and Erk-Abl (19–21). These studies indicate that STAT5a and STAT5b are involved in many different signaling pathways. Gene disruption of individual genes in mice revealed that both STAT5a and STAT5b play essential but often redundant roles in the physiological responses associated with PRL (22). Although they share high homology, it is also reported that STAT5a and STAT5b may be differentially activated (23) and bind DNA sequences with distinct specificities (24). STAT5 undergoes a rapid and transient activation and deactivation cycle through tyrosine phosphorylation upon cytokine stimulation (25). It is generally thought that phosphatases attenuate or block tyrosine phosphorylation-mediated signals and play a negative role. However, since the finding that dephosphorylation of c-Src leads to the activation (26), it is conceivable that the phosphatases could also play a positive role in some signaling cascades. Actually, one of the SH2-containing protein-tyrosine phosphatases (PTPs), SHP-2, was shown to be

* This work was supported in part by grants from Japan Society for Bioscience, Biotechnology, and Agrochemistry. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Applied Molecular Biosciences, Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan. Fax: 81-52-789-4128; E-mail: naoki@agr.nagoya-u.ac.jp.
Dephosphorylation and Deactivation of STAT5 by PTP1B

essential for interferon α/β-induced gene transcription (27), and recent publications have shown that SHP-2 contributes to β-casein promoter activation in a positive manner (28, 29). However, dephosphorylation of the activated JAK2 and STAT5 through the PRL receptor and the involvement of the PTPs in a negative regulation have poorly been understood.

Recently, we found that 16 different PTPs including SHP-1 and SHP-2 were expressed in mammary glands and mammary epithelial cells and that most of them were down-regulated in lactating mammary glands (30). To extend these findings, in this study we investigated the involvement of each PTP in PRL receptor-mediated signaling pathway by using expression constructs for prolactin receptor, STAT5a/b, and each PTP, and we found that both of the prolactin-induced tyrosine phosphorylations of STAT5a and STAT5b and promoter activation of β-casein gene were abolished in COS7 cells when cytosolic PTP1B was overexpressed. Nuclear translocation of STAT5 was also inhibited by PTP1B. Overexpression of PTP1B in mammary epithelial COMMA-1D cells also resulted in dephosphorylation of PRL-activated STAT5 and down-regulation of β-casein gene expression upon lactogenic hormone treatment. STAT5a and STAT5b were dephosphorylated by recombinant PTP1B in vitro and were co-immunoprecipitated by substrate-trapping mutants of PTP1B. These results strongly suggest that STAT5a and STAT5b are specific substrates of PTP1B and are deactivated by the phophatase in PRL-mediated signaling pathway.

EXPERIMENTAL PROCEDURES

Materials, Antibodies, and Plasmid Constructs—Ovine prolactin (PRL) used for cell treatment was obtained from Sigma. Recombinant antibodies to STAT5 (C-17), recognizing both mSTAT5a and mSTAT5b, and Myc epitope (9E10) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse phospho-tyrosine antibodies (4G10) were purchased from Biosource Inc. (Lake Placid, NY). Monoclonal antibodies to STAT5 were obtained from Sigma. Protein A-Sepharose beads used for immunoprecipitation were obtained from Sigma. HA epitope (Y-11), and Myc epitope (9E10) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). HA-recognition polyclonal antibodies were purchased from Sigma. Ovine prolactin was purchased from Ovine prolactin, Inc. (Helena, MT). HA tagging to PTP1B at its N terminus was done by PCR amplification using 5'-CCA-AGT-ACT-AGC-CTG-AGA-TGG-AG-3' and 3'-GAC-TCC-AAA-GTG-CAG-CAT-5'.

Cell Culture and Transfection—Cell lines were cultured and grown overnight in DMEM containing 10% FCS. Upon transfection experiments, COS7 cells were inoculated at a density of 2 × 10⁴ cells/6-cm dish and grown overnight in DMEM containing 10% FCS. Expression plasmids were transfected into the cells by the modified calcium phosphate precipitation method (33). After incubation under 3% CO₂, 97% air for 18 h, the transfected cells were washed with phosphate-buffered saline twice and cultured in fresh DMEM containing 10% FCS for another 24 h under humidified 5% CO₂ and 95% air. Prior to PRL stimulation (5 μg/ml), cells were serum-starved for 16 h. Luciferase and β-galactosidase activities were determined as described (29).

Retrovirus-mediated Gene Delivery—HA-tagged PTP1B was ligated into pLXSN retroviral vector (CLONTECH) via EcoRI site and introduced into Phoenix ecotropic packaging cells, which were obtained from Dr. Garry P. Nolan (Stanford University), by the modified calcium phosphate precipitation method (35). COMMA-1D cells were infected with the retrovirus-containing culture medium and then selected in the presence of G418 (1 mg/ml). To eliminate clonal deviation, G418-resistant polyclonal cells were used for experiments.

Cell Lysis and Western Blotting—The transfected cells were lysed with “lysis buffer” containing 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 10 mM sodium phosphate, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin. Proteins in the cell lysate were separated by SDS-PAGE under reducing conditions according to the method of Laemmli (34) followed by blotting onto nitrocellulose membranes (Hybond C+, Amersham Pharmacia Biotech). The membranes were blocked in NET buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.05% Triton X-100) containing 1% gelatin and then sequentially incubated with the respective antibodies and horseradish peroxidase-conjugated goat anti-rabbit or -mouse (Bio-Rad). The protein bands were visualized with an enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia Biotech). In Vitro Dephosphorylation Assay— GST fusion proteins containing full-length PTPs, SHP-2, and SHP-1 were used for dephosphorylation assays. GST antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). GST fusion proteins containing the cysteine to serine alteration at position 215 and a aspartic acid to asparagine alteration at position 373 were employed using COS7 cells. With this strategy, SHP-2 was inhibited by PTP1B. Overexpression of PTP1B in mammary epithelial COMMA-1D, HC11, and primary mammary epithelial cell lines COMMA-1D, HC11, and primary mammary epithelial cells are quite resistant to gene transfection. Therefore, to study the involvement of each PTP identified in PRL receptor-mediated signaling, co-transfection strategy was employed using COS7 cells. With this strategy, SHP-2 was shown to be a positive regulator of PRL receptor-mediated signal transduction pathway (29). Among the 16 PTPs identified (30), the following 12 PTPs were examined: PTP1B, SHP-1, SHP-2, PTP36, HCSF, PTP-BAS, PTPα, PTPδ, PTPβ, and PTPρ. COS7 cells were triple co-transfected with the expression plasmids for PRL receptor, STAT5a, and the respective PTPs and were stimulated with PRL following serum starvation. STAT5a was immunoprecipitated, and its tyrosine phosphorylation level was assessed by immunoblotting with the anti-phosphotyrosine antibody. As shown in Fig. 1, A and B, strong tyrosine phosphorylation was caused upon PRL stimulation when no PTPs were transfected. When a cytosolic
protein tyrosine phosphatases PTP1B was co-transfected, tyrosine phosphorylation of STAT5a was strongly reduced and nearly undetectable. Although the expression level of other PTPs was obvious (Fig. 1C), the other PTPs including SHP-1 and SHP-2, on the other hand, exhibited apparently no effect on the tyrosine phosphorylation of STAT5a, which was consistent with previous reports (29).

PTP1B Dephosphorylates PRL-activated STAT5a and STAT5b—To confirm the possible dephosphorylation of STAT5 by PTP1B, catalytically inactive mutants of PTP1B were constructed and co-transfected into COS7 cells with PRL receptor and STAT5a or STAT5b. Upon overexpression of PTP1B wild-type, PRL-induced tyrosine phosphorylation of both STAT5a and STAT5b was largely abolished (Fig. 2A). Dephosphorylation activity was not observed when the cells were co-transfected with catalytically inactive Cys/Ser and Asp/Ala mutants of PTP1B (Fig. 2A), suggesting that phosphatase activity is essential for the dephosphorylation of STAT5 proteins.

To relate the expression level of PTP1B with the dephosphorylation of STAT5a and STAT5b, various amounts of expression plasmids for PTP1B were co-transfected into COS7 cells, and tyrosine phosphorylation of STAT5a and STAT5b was assessed. Approximately 80% of STAT5a was dephosphorylated when 0.1 μg of PTP1B was co-transfected (Fig. 2B, lane 3), and transfection with higher amounts of plasmid (1 and 2 μg) abolished tyrosine phosphorylation of STAT5a (Fig. 2B, lanes 2 and 1, respectively). Nearly the same dephosphorylation of STAT5b was caused by expression of PTP1B wild-type in a manner dependent of its expression level (Fig. 2B, lanes 5–8).

Prolactin binding to its cognate receptor results in the autophosphorylation and activation of JAK2, and in turn JAK2 tyrosine phosphorylates both STAT5a and STAT5b. Tyrosine phosphorylation of JAK2 is essential for its full activation (5). If PTP1B dephosphorylates JAK2, tyrosine phosphorylation of STAT5a and STAT5b should be reduced accordingly. To know direct or indirect dephosphorylation activity of PTP1B against STAT5, COS7 cells were co-transfected with PRL receptor and PTP1B. Cells were serum-starved, stimulated with PRL for 30 min, lysed, and then subjected to immunoprecipitation with anti-HA (for PTP1B, SHP-1, PTP6, HCSF, PTP-BAS, PTPe, PTPx, LAR, PTPγ, and PTP1), anti-FLAG (for PTPλ), and anti-Myc (for SHP-2) antibodies.

**Fig. 1.** Identification of PTPs that dephosphorylate prolactin-induced tyrosine-phosphorylated STAT5a. COS7 cells were transiently co-transfected with expression plasmids for prolactin receptor (1 μg), STAT5a (1 μg), and empty vector (mock) or each PTP indicated (2 μg for each). A, following starvation cells were left untreated (-) or stimulated (+) with PRL (5 μg/ml) for 30 min and lysed followed by immunoprecipitation with anti-STAT5 antibody. Immunoprecipitates were separated by SDS-PAGE (10% gel), transferred to a nitrocellulose membrane, and probed with anti-phosphotyrosine antibody (upper panel). The membrane was stripped and reprobed with anti-STAT5 antibody (lower panel). B, tyrosine phosphorylation level of STAT5a was densitometrically normalized. Phosphorylation level of STAT5a in mock transfectant stimulated with PRL was set as 100%. Mean values and S.D. of three independent experiments are shown. C, an aliquot of the total cell lysates was immunoblotted with a mixture of anti-HA (for PTP1B, SHP-1, PTP6, HCSF, PTP-BAS, PTPe, PTPx, LAR, PTPγ, and PTP1), anti-FLAG (for PTPλ), and anti-Myc (for SHP-2) antibodies.
Dephosphorylation and Deactivation of STAT5 by PTP1B

Fig. 2. Dephosphorylation of STAT5a and STAT5b by PTP1B in transfected COS7 cells. A, COS7 cells were co-transfected with expression plasmids for PRL receptor (2 μg), STAT5a or STAT5b (1 μg), and empty vector (mock) or each C/S or D/A mutant (2 μg for each). Following serum starvation, cells were left untreated (−) or stimulated (+) with PRL (5 μg/ml) for 30 min and lysed. STAT5a and STAT5b were immunoprecipitated (IP), separated on SDS-PAGE, and transferred to a nitrocellulose membrane, and probed with anti-phosphotyrosine antibody (αPY) (4G10). The same blot was reprobed with anti-STAT5 antibody (αSTAT5). B, COS7 cells were co-transfected with expression plasmids for PRL receptor (1 μg), STAT5a or STAT5b (1 μg), and varying amounts of HA-PTP1B wild-type as indicated and stimulated with PRL (5 μg/ml) for 30 min and lysed. STAT5a and STAT5b were immunoprecipitated (IP), separated on SDS-PAGE, transferred to a nitrocellulose membrane, and probed with anti-phosphotyrosine antibody (αPY). The membrane was stripped and reprobed with anti-HA antibody (αHA). C, COS7 cells were co-transfected with expression plasmids for PRL receptor (2 μg) and empty vector (mock) or each of HA-PTP1B wild-type, catalytically inactive Cys/Ser (C/S) and Asp/Ala (D/A) mutants (2 μg for each). Following serum starvation, cells were left untreated (−) or stimulated (+) with PRL (5 μg/ml) for 30 min and lysed. STAT5a and STAT5b were immunoprecipitated (IP), separated on SDS-PAGE, and immunoblotted with anti-phosphotyrosine antibody. The membrane was stripped and reprobed with anti-JAK2 antibody.

Dephosphorylation and Deactivation of STAT5 by PTP1B

We then examined the kinetics of STAT5a and STAT5b dephosphorylation by PTP1B. Transfected COS7 cells were serum-starved and stimulated with PRL for 2–60 min. STAT5a and STAT5b were immunoprecipitated and assessed by phosphotyrosine immunoblotting. As shown in Fig. 4A, PRL induced rapid tyrosine phosphorylation of STAT5a and STAT5b within 5 min in mock-transfected COS7 cells, and the levels of tyrosine phosphorylation were kept high even 60 min after PRL stimulation. This time course of tyrosine phosphorylation of STAT5a was quite similar to endogenous STAT5a/b of mammary epithelial COMMA-1D cells (Fig. 4B). By overexpressing PTP1B wild-type, dephosphorylation of STAT5a and STAT5b already occurred 5 min after PRL stimulation, and faint signals were detected 30 and 60 min after PRL stimulation. PTP1B Inhibited Nuclear Translocation of STAT5a and STAT5b—It has been reported that PTP1B is localized in endoplasmic reticulum through its C-terminal hydrophobic amino acid residues (37). To address the possibility that STAT5 might be dephosphorylated by PTP1B in cytosol, subcellular localization of STAT5a and STAT5b following PRL stimulation was dicated amounts of the recombinant GST-PTP1B fusion proteins were added to the immune complexes and incubated at 37 °C for 30 min. As clearly illustrated in Fig. 4A, tyrosine phosphorylation level of STAT5a was reduced to approximately 50% by 1 μg of GST-PTP1B wild-type and incubation with 10 μg of the fusion protein resulted in complete dephosphorylation of STAT5a (upper panels). In a similar manner, STAT5b was dephosphorylated by GST-PTP1B wild-type (lower panels). Incubation of the immune complexes with empty GST and GST fused to catalytically inactive mutants of PTP1B resulted in no reduction in tyrosine phosphorylation level of STAT5a and STAT5b. In addition, phosphorylated JAK2 was immunoprecipitated from PRL-stimulated cells with anti-jak2 antibody and incubated with 10 μg of GST, GST-PTP1B wild-type, or its mutants as above. No tyrosine dephosphorylation was observed, which is consistent with the in vitro data shown in Fig. 2C.
PX was transfected into COS7 cells together with the PRL receptor and STAT5a or STAT5b, and the \( \beta \)-casein gene promoter-luciferase construct. A \( \beta \)-galactosidase gene was also included to normalize for transfection efficiency. Luciferase activity was determined in extracts from cells left untreated or stimulated with PRL. As shown in Fig. 6A, both STAT5a and STAT5b together with the PRL receptor could strongly activate the \( \beta \)-casein gene promoter upon PRL stimulation of the cells that had been mock-transfected with empty plasmid without PTP (lanes 1, 2, 9, and 10). When PTP1B wild-type was co-expressed, such transcriptional induction was completely suppressed (lanes 4 and 12), whereas PTP1B Cys/Ser and Asp/Ala mutants exhibited slightly higher \( \beta \)-casein promoter activation as compared with mock transfectants (lanes 6, 8, 14, and 16). Furthermore, expression of PTP1B wild type caused a dose-dependent suppression of transactivation of STAT5a and STAT5b by PRL (Fig. 6B). These results indicate that PTP1B is a negative regulator of PRL-mediated \( \beta \)-casein promoter activation, by dephosphorylating STAT5a and STAT5b.

PTP1B \textit{Is a Negative Regulator in PRL Receptor-mediated Signaling in Mammary Epithelial Cells}—As mentioned, mammary epithelial cells are quite resistant to gene transfection. Therefore, it was required to use virus-mediated gene infection strategy. PTP1B CDNA was ligated into a retroviral vector and introduced into mammary epithelial COMMA-1D cells. Cells were selected in cell culture medium containing G418 and then directly used for experimentation. As shown in Fig. 7A, nearly the same amounts of HA-PTP1B wild type, Cys/Ser, and Asp/Ala were expressed in cells. Cells were serum-starved and stimulated with PRL. Endogenous STAT5 was immunoprecipitated and assayed by immunoblotting with anti-phosphotyrosine antibody. As was seen in reconstituted COS7 cells, phosphorylation level of JAK2 (Fig. 7C). Moreover, \( \beta \)-casein gene expression was suppressed in PTP1B wild type-expressing COMMA-1D cells, whereas that in PTP1B mutant-expressing cells was comparable to that in mock-infected cells (Fig. 7D). These clearly indicate that PTP1B is a negative regulator in PRL receptor-mediated signaling pathway in mammary epithelial cells.

\textbf{STAT5 Is a Specific Substrate of PTP1B}—To confirm further that STAT5 is a specific substrate of PTP1B, a co-precipitation study was carried out using recombinant GST-PTP1B fusion proteins. COMMA-1D cells were stimulated with PRL for 30 min following serum starvation and lysed. The cell lysates were mixed with 10 \( \mu \)g of empty GST or GST-PTP1B fusion proteins and were being rocked together with GSH-Sepharose beads at 4 \(^\circ\)C for 3 h. The GSH-Sepharose beads were washed with the lysis buffer and dissolved in SDS sample buffer. Proteins were separated by SDS-PAGE (10\% gel) and blotted onto nitrocellulose membranes. The membranes were probed with anti-phosphotyrosine antibody. As shown in Fig. 8A, many tyrosine-phosphorylated proteins including a 97-kDa protein were detected in PTP1B wild-type-expressing COMMA-1D cells whereas that in PTP1B mutant-expressing 97-kDa band co-precipitated with the PTP1B Asp/Ala mutant. Therefore, it was required to use virus-mediated gene infection strategy. PTP1B CDNA was ligated into a retroviral vector (mock) or HA-PTP1B wild type (2 \( \mu \)g for each). Following serum starvation, cells were left untreated (0 min) or stimulated with PRL (5 \( \mu \)g/ml) for the indicated times following PRL stimulation for the times indicated, and STAT5 proteins were immunoprecipitated and immunoblotted with anti-STAT5 antibody. The tyrosine-phosphorylated proteins including a 97-kDa protein were co-precipitated with anti-STAT5 antibody (Fig. 8A, lower panel). As was seen in reconstituted COS7 cells, phosphorylation level of JAK2 (Fig. 7C). Moreover, \( \beta \)-casein gene expression was suppressed in PTP1B wild type-expressing COMMA-1D cells, whereas that in PTP1B mutant-expressing cells was comparable to that in mock-infected cells (Fig. 7D). These clearly indicate that PTP1B is a negative regulator in PRL receptor-mediated signaling pathway in mammary epithelial cells.
with GST fusion proteins have not been well characterized so far. To confirm that, COS7 cells, which had been co-transfected with PRL receptor and STAT5a or STAT5b, were stimulated with PRL and the lysates were precipitated with the GST-PTP1B fusion proteins as above. As clearly shown in Fig. 8B, phosphorylated STAT5a or STAT5b was detected in the precipitates of the Asp/Ala and to a lesser extent in Cys/Ser mutants, whereas no signal was observed in the precipitates of the wild type. The immunoblotting with anti-STAT5 also showed that the Asp/Ala mutant precipitated the STAT5 most strongly. However, the anti-STAT5 immunoblotting also revealed that STAT5a and STAT5b were co-precipitated by not only substrate-trapping mutants of PTP1B but also the wild type, suggesting some contribution of phosphotyrosine-independent interaction between STAT5 and PTP1B.

**DISCUSSION**

Although the mechanisms how STAT proteins become activated have been well characterized, much less is known about their subsequent deactivation process. Recent publications have focused on the negative regulation of STAT proteins, and several mechanisms have been documented for deactivation of STAT5. A family of JAK kinase-binding protein or cytokine-inducible SH2-containing protein has been shown to down-regulate STAT5 by inhibiting upstream JAK kinase activity or preventing recruitment of STAT5 to cytokine receptors (38–43). It has also been demonstrated that the function of STAT5 can be modulated by the ubiquitin-proteasome pathway (25, 44–46). Although tyrosine phosphorylation of STAT5 is the essential step for its full biological functions (36), dephosphorylation of STAT5 has been largely unknown.

SHP-2 and structurally related SHP-1 have been shown to be widely implicated in distinct JAK-STAT pathways. Positive involvement of SHP-2 in PRL receptor-mediated signaling pathway has been shown by two groups (28, 29). Upon PRL stimulation, SHP-2 forms a trimeric complex with PRL receptor and JAK2, and SHP-2 itself becomes tyrosine-phosphorylated. Catalytically inactive mutants of SHP-2 inhibited the signaling pathway in a dominant negative fashion, suggesting that the phosphatase activity of SHP-2 is essential, possibly through dephosphorylating essential tyrosine residues on JAK2 for its full activation (29). SHP-2 has also been shown to be involved positively in IL-2-mediated signaling in NK cells (47), whereas it plays a negative role in gp130 signaling (48). It is also reported that SHP-1 is activated upon growth hormone stimulation and might dephosphorylate STAT5b in rat liver cells (49). Thus, SHP-2 and SHP-1 might play a positive or negative role depending on the cells and tissue where they express.

In the present study, we showed that a cytosolic phosphatase PTP1B dephosphorylated PRL-activated STAT5a and STAT5b.
in transfected COS7 cells as well as in vitro. In contrast, other cytoplasmic PTPs, especially SHP-1 and SHP-2, and receptor type PTPs had no effect on the tyrosine phosphorylation level of STAT5a (Fig. 1) and STAT5b (data not shown). Further detailed analyses revealed that catalytic activity of PTP1B is essential for the dephosphorylation of STAT5a and STAT5b and that PTP1B directly dephosphorylated STAT5a and STAT5b but not JAK2, upstream regulator of STAT5 (Figs. 2 and 3). Additionally, tyrosine-phosphorylated STAT5a and STAT5b were co-precipitated with the substrate-trapping mutants of PTP1B (Fig. 8), and PRL-induced transcriptional activation of STAT5a and STAT5b was disrupted when PTP1B was overexpressed (Fig. 6). Moreover, PTP1B was shown to be a negative regulator in PRL receptor-mediated signaling pathway leading to up-regulation of β-casein gene expression in mammary epithelial COMMA-1D cells (Fig. 7). These results strongly and clearly suggest that PTP1B is a principal PTP specifically dephosphorylating and deactivating PRL-activated STAT5a and STAT5b.

Our previous data showed that expression of PTP1B was largely suppressed in lactating mammary gland producing a huge amount of milk proteins under the control of PRL and that the expression level returned to that in virgin mammary gland immediately after suckling cessation and weaning of...
incubated with GST fusion proteins and processed as above. Partially with anti-phosphotyrosine antibody (4G10) and anti-STAT5 antibody. Precipitates were washed with lysis buffer, separated on SDS-PAGE, transferred onto nitrocellulose membranes, and probed sequentially with anti-phosphotyrosine antibody (4G10) and anti-STAT5 antibody. B, PRL receptor (2 µg) and STAT5a or STAT5b were co-expressed in COS7 cells, and the cells were serum-deprived and incubated with PRL (5 µg/ml) for 30 min, and histones were extracted with GST fusion proteins and analyzed by Western blotting. As shown in Figure 8, PRL receptor Nib2 form (56). Substitution of tyrosine 382 with alanine on the receptor did not affect tyrosine phosphorylation of STAT5, but both nuclear translocation and binding to the target DNA sequence were inhibited. This suggests potential involvement of PTPs, which dephosphorylate the tyrosine residues on the PRL receptor, in PRL-mediated target gene activation. Indeed, we have observed a receptor-type phosphatase PTPα dramatically inhibited PRL-mediated β-casein promoter activation without affecting the phosphorylation level of STAT5.2

In conclusion, we demonstrated in this study for the first time the specific tyrosine dephosphorylation of PRL-induced STAT5 by cytoplasmic PTP1B. We further reported that nuclear translocation and transcriptional activities of STAT5 were largely inhibited. In addition to STAT5, it has been shown that the PRL receptor-mediated signaling cascade also results in tyrosine phosphorylation of STAT1 and STAT3 (57). Whether PTP1B is also involved in negative regulation in other JAK-STAT pathways is currently in progress in our laboratory.

Acknowledgments—We thank Drs. Berund Groner, James N. Ihle, Carl-Henrik Heldin, Hitoshi Miyazaki, Axel Ullrich, Hava Abraham, and Masato Ogata for provision of expression plasmids and antibodies. We also thank Dr. Garry P. Nolan for provision of Phoenix ecotropic packaging cells.

PTP1B was originally purified from the cytosolic fraction of human placenta as a 37-kDa protein (51) and is now known to be ubiquitously and abundantly expressed in various eukaryotic cells and be associated with endoplasmic reticulum and be translocated to the plasma membrane (52). We showed that most of exogenously expressed PTP1B was recovered in cytosol, and its location, amount, and size were unchanged following PRL stimulation. Constitutive existence of endogenous PTP1B in cytosol might grant concomitant tyrosine dephosphorylation of STAT5.

It has been reported that PTP1B is serine-phosphorylated upon 12-0-tetradecanoylphorbol-13-acetate treatment through the action of protein kinase C (53) and CLK1 and CLK2 (54) and that PTP1B undergoes tyrosine phosphorylation upon epidermal growth factor stimulation (55). In our examination, no phosphorylation of tyrosine and serine/threonine residues was detected by immuno blotting analysis using anti-phosphotyrosine and anti-phosphoserine/threonine antibodies (data not shown). Whether modulation of PTP1B occurs after PRL stimulation remains to be determined.

Recently, Ali and Ali (56) have reported that STAT5 tyrosine phosphorylation and nuclear translocation are regulated by two separate pathways by using a variety of mutants of the PRL receptor Nib2 form (56). Substitution of tyrosine 382 with alanine on the receptor did not affect tyrosine phosphorylation of STAT5, but both nuclear translocation and binding to the target DNA sequence were inhibited. This suggests potential involvement of PTPs, which dephosphorylate the tyrosine residues on the PRL receptor, in PRL-mediated target gene activation. Indeed, we have observed a receptor-type phosphatase PTPα dramatically inhibited PRL-mediated β-casein promoter activation without affecting the phosphorylation level of STAT5.2

In conclusion, we demonstrated in this study for the first time the specific tyrosine dephosphorylation of PRL-induced STAT5 by cytoplasmic PTP1B. We further reported that nuclear translocation and transcriptional activities of STAT5 were largely inhibited. In addition to STAT5, it has been shown that the PRL receptor-mediated signaling cascade also results in tyrosine phosphorylation of STAT1 and STAT3 (57). Whether PTP1B is also involved in negative regulation in other JAK-STAT pathways is currently in progress in our laboratory.

Acknowledgments—We thank Drs. Berund Groner, James N. Ihle, Carl-Henrik Heldin, Hitoshi Miyazaki, Axel Ullrich, Hava Abraham, and Masato Ogata for provision of expression plasmids and antibodies. We also thank Dr. Garry P. Nolan for provision of Phoenix ecotropic packaging cells.

PTP1B was originally purified from the cytosolic fraction of human placenta as a 37-kDa protein (51) and is now known to be ubiquitously and abundantly expressed in various eukaryotic cells and be associated with endoplasmic reticulum through its C-terminal hydrophobic 35-amino acid region (37), although there is also evidence indicating its association with the plasma membrane (52). We showed that most of exogenously expressed PTP1B was recovered in cytosol, and its location, amount, and size were unchanged following PRL stimulation. Constitutive existence of endogenous PTP1B in cytosol might grant concomitant tyrosine dephosphorylation of STAT5.

It has been reported that PTP1B is serine-phosphorylated upon 12-0-tetradecanoylphorbol-13-acetate treatment through the action of protein kinase C (53) and CLK1 and CLK2 (54) and that PTP1B undergoes tyrosine phosphorylation upon epidermal growth factor stimulation (55). In our examination, no phosphorylation of tyrosine and serine/threonine residues was detected by immuno blotting analysis using anti-phosphotyrosine and anti-phosphoserine/threonine antibodies (data not shown). Whether modulation of PTP1B occurs after PRL stimulation remains to be determined.

Recently, Ali and Ali (56) have reported that STAT5 tyrosine phosphorylation and nuclear translocation are regulated by two separate pathways by using a variety of mutants of the PRL receptor Nib2 form (56). Substitution of tyrosine 382 with alanine on the receptor did not affect tyrosine phosphorylation of STAT5, but both nuclear translocation and binding to the target DNA sequence were inhibited. This suggests potential involvement of PTPs, which dephosphorylate the tyrosine residues on the PRL receptor, in PRL-mediated target gene activation. Indeed, we have observed a receptor-type phosphatase PTPα dramatically inhibited PRL-mediated β-casein promoter activation without affecting the phosphorylation level of STAT5.2

In conclusion, we demonstrated in this study for the first time the specific tyrosine dephosphorylation of PRL-induced STAT5 by cytoplasmic PTP1B. We further reported that nuclear translocation and transcriptional activities of STAT5 were largely inhibited. In addition to STAT5, it has been shown that the PRL receptor-mediated signaling cascade also results in tyrosine phosphorylation of STAT1 and STAT3 (57). Whether PTP1B is also involved in negative regulation in other JAK-STAT pathways is currently in progress in our laboratory.
Dephosphorylation and Deactivation of STAT5 by PTP1B

(1999) J. Biochem. (Tokyo) 125, 669–675
31. Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 488–492
32. Ayama, K., Matsuda, T., and Aoki, N. (1999) Biochem. Cell Biol. 76, 523–531
33. Chen, C., Okayama, H. (1987) Mol. Cell. Biol. 7, 2745–2752
34. Laemmli, U. K. (1970) Nature 227, 680–685
35. Aoki, N., Yamaguchi-Aoki, Y., and Ullrich, A. (1996) J. Biol. Chem. 271, 29422–29426
36. Gouilleux, F., Wakao, H., Mundt, M., and Groner, B. (1994) EMBO J. 13, 4361–4369
37. Frangioni, J. V., Beahm, P. H., Shifrin, V., Jost, C. A., and Neel, B. G. (1992) Cell 68, 545–560
38. Aman, M. J., Migone, T.-S., Sasaki, A., Ascherman, D. P., Zhu, M., Soldaini, E., Imada, K., Miyajima, A., Yoshimura, A., and Leonard, W. J. (1999) J. Biol. Chem. 274, 30266–30272
39. Cohney, S. J., Sanden, D., Cacalano, N. A., Yoshimura, A., Mui, A., Migone, T. S., and Johnstone, J. A. (1999) Mol. Cell. Biol. 19, 4980–4988
40. Tomic, S., Chughtai, N., and Ali, S. (1999) Mol. Cell. Endocrinol. 158, 45–54
41. Helman, D., Sadowski, Y., Cohen, Y., Matsumoto, A., Yoshimura, A., Merchav, S., and Gertler, A. (1998) FEBS Lett. 441, 287–291
42. Verdier, F., Chretien, S., Muller, O., Varlet, P., Yoshimura, A., Gisselbrecht, S., Lacombe, C., and Mayeux, P. (1998) J. Biol. Chem. 273, 26185–26190
43. Matsumoto, A., Masuhara, M., Mitsui, K., Yokouchi, M., Obtsuto, M., Misawa, H., Miyajima, A., and Yoshimura, A. (1997) Blood 89, 3145–3154
44. Callus, B. A., and Mathey-Prevot, B. (1998) Blood 91, 3182–3192
45. Wang, D., Morigli, R., Stravopodis, D., Carpino, N., Marine, J.-C., Teglund, S., Feng, J., and Igle, J. N. (2000) EMBO J. 19, 392–399
46. Gadina, M., Stanco, L. M., Bacon, C. M., Larner, A. C., and O'Shea, J. J. (1998) J. Immunol. 160, 4657–4661
47. Syms, A., Stahl, N., Reeves, S. A., Farrugella, T., Servidei, T., Gearan, T., Yancopoulos, G., and Fink, J. S. (1997) Curr. Biol. 7, 697–700
48. Ram, P. A., and Waxman, D. J. (1997) J. Biol. Chem. 272, 17694–17702
49. Haspel, R. L., Salditt-Georgieff, M., and Darnell, J. E., Jr. (1996) EMBO J. 15, 6292–6298
50. Tonks, N. K., Diltz, C. D., and Fischer, E. H. (1988) J. Biol. Chem. 263, 6722–6730
51. Liu, F., Hill, D. E., and Chernoff, J. (1996) J. Biol. Chem. 271, 31290–31295
52. Flint, A., Geblen, M. F. G. B., Franza, B. R., Hill, D. E., Jr., and Tonks, N. K. (1993) EMBO J. 12, 1937–1946
53. Moelein, F. M., Myers, M. P., Landreth, G. E. (1999) J. Biol. Chem. 274, 26697–26704
54. Liu, F., and Chernoff, J. (1997) Biochem. J. 327, 139–145
55. Ali, S., and Ali, S. (1998) J. Biol. Chem. 273, 7709–7716
56. DaSilva, L., Rui, H., Erwin, R. A., Howard, O. M., Kirken, R. A., Malaharba, M. G., Hacket, R. H., Larner, A. C., and Farrar, W. L. (1996) Mol. Cell. Endocrinol. 117, 131–149

WITHDRAWN
February 15, 2011