SHP-2 Regulates SOCS-1-mediated Janus Kinase-2 Ubiquitination/Degradation Downstream of the Prolactin Receptor*

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The protein tyrosine phosphatase SHP-2 is an important regulator of the Janus kinase-2 (Jak2)/signal transducer and activator of transcription (Stat) pathway downstream of the cytokine/prolactin receptor family. We report that SHP-2 dephosphorylates tyrosine (Tyr-1007) of Jak2 kinase, a critical recruitment site for the ubiquitin ligase-associated inhibitory protein suppressor of cytokine signaling-1 (SOCS-1), thereby contributing to Jak2 stability. Inactivation of SHP-2 function by blocking receptor/SHP-2 association or by using a catalytically inactive mutant of SHP-2 led to a marked increase in Jak2 ubiquitination/degradation, Jak2 phosphorylation on Tyr-1007, and Jak2/SOCS-1 association. Furthermore, functional studies indicate that modulating the interaction of Jak2/SOCS-1 by SHP-2 is essential for prolactin/Stat5-mediated signaling. Together our results provide a novel function for SHP-2 as a positive regulator of cytokine receptor signaling by regulating ubiquitination/degradation pathways.

Prolactin (PRL) is a polypeptide neuroendocrine hormone that exerts a broad range of biological effects on diverse target tissues. PRL regulates different biological processes such as those involved in mammary gland development, reproduction, and immune regulation (reviewed by Ref. 1). The hormone generates its multiple biological functions by interacting with the PRL receptor (PRLR), a member of the class I cytokine receptor superfamily (2). Ligand binding to the PRLR induces receptor dimerization leading to activation of the constitutively associated Janus kinase-2 (Jak2), resulting in Jak2 and receptor tyrosine phosphorylation (3, 4). These membrane-proximal signaling events play a critical role in signal propagation by recruiting effector molecules such as the signal transducer and activator of transcription-5 (Stat-5) (5). The Jak2/Stat-5 pathway has been shown to be indispensable in mediating PRL responses, leading to various physiological responses such as terminal differentiation of mammary epithelial cells (6).

SHP-2, a ubiquitously expressed cytoplasmic protein tyrosine phosphatase, plays a central role in signaling downstream of receptor tyrosine kinases, G-protein-coupled receptors, and cytokine receptors including the PRLR (7). Following PRLR activation, SHP-2 is tyrosine-phosphorylated, physically associates to the carboxyl-terminal tyrosine of the PRLR, and is critical for activation of Stat5 and induction of the β-casein gene promoter (4, 8–10). The precise function of SHP-2 in PRLR signaling thus is of vital importance and has yet to be fully characterized.

Modulation of Jak kinase activity has appeared as an important mechanism of regulation of cytokine receptor signaling. Studies have indicated that protein tyrosine phosphatases and adaptor/proteasomal degradation machinery are two means by which Jak2 kinase activity is regulated downstream of cytokine receptors. The protein tyrosine phosphatase SHP-1 (11, 12), CD45 (13), and PTP1B (14–17) were shown to dephosphorylate Jak2, and block cytokine receptor signaling. In the case of CD45 and PTP1B, it was demonstrated that these phosphatases inhibit the activation of Jak2 through dephosphorylating Tyr-1007 within the activation loop of the kinase (18).

The suppressor of cytokine signaling-1 (SOCS-1, also referred to as JAB) (19, 20) has been established as an important negative-feedback inhibitor of cytokine-activated Jak/Stat signaling pathway (reviewed in Refs. 21 and 22). SOCS-1 is a member of the SOCS family of proteins that includes eight members, SOCS-1 to SOCS-7 and cytokine-inducible SH2-containing protein. Similar to other members of the family, SOCS-1 contains a central SH2 domain followed by a conserved carboxyl-terminal region called the SOCS-box. The inhibitory role of SOCS-1 is in part due to the fact that Jak2/SOCS-1 interaction blocks substrate access to Jak2 kinase through a 24 amino acid segment denoted as the kinase inhibitory region (22, 24). Recruitment of SOCS-1 through its SH2 domain to phosphorylated Tyr-1007 in the activation loop of Jak2 leads to inhibition of the catalytic activity of Jak2. A second critical role for SOCS-1 in down-regulating signaling is through targeting associated proteins to proteasomal degradation. Various studies have indicated that this function of SOCS-1 is attributed to the highly conserved SOCS-box (25–27). The SOCS-box of SOCS-1 was shown to target both the fusion product TEL (the Ets-variant gene 6)-Jak2 (28, 29) and the full-length wild type Jak2 (30) to ubiquitination and proteasomal degradation. This degradation process was dependent on Jak2 phosphorylation on Tyr-1007.

In this study, we investigated the hypothesis that SHP-2 contributes to PRL-mediated activation of the Jak2/Stat5 pathway by modulating the formation of a complex between Jak2 and SOCS-1, thus maintaining the activity and stability of Jak2. We determined that aborting SHP-2 activity downstream of the

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1 The abbreviations used are: PRL, prolactin; PRLR, PRL receptor; WT, wild type; Jak2, Janus kinase-2; Stat, signal transducer and activator of transcription; SOCS-1, suppressor of cytokine signaling-1; SH2, Src homology 2; FBS, fetal bovine serum; SH2, SH2 domain-containing protein tyrosine phosphatase-2; PTP1B, protein tyrosine phosphatase-1B; CA, catalytically inactive C463A mutant form of SHP-2.
PRL by either using a mutant form of the PRLR, deficient in
SHP-2 recruitment site, or by using a phosphatase inactive mu-
tant of SHP-2 resulted in a significant increase in Jak2 phos-
phorylation on Tyr-1007 and in the formation of a Jak2/SOCS-1
complex, in turn leading to the ubiquitination and degrada-
tion of Jak2. Therefore, SHP-2 critically regulates PRLR signal-
ing by dephosphorylating SOCS-1 recruitment site within Jak2,
conse-
quently allowing signal propagation/maintenance.

EXPERIMENTAL PROCEDURES

Materials, Plasmid Constructs, and Antibodies—Expression plas-
mids encoding the Nb2 form of the PRLR and the corresponding COOH-
terminal tyrosine to phenylalanine mutant form, Nb2PRLRY382F, as
well as Jak2 were described previously (4). Expression plasmids encod-
ing the wild type form of SHP-2 and the catalytically inactive cytokine
463 to alanine mutant SHP-2CA were obtained from Axel Ulrich (Max-
Plank Institute, Germany). Expression plasmids encoding Myc-SOCS-1
and an SH2 domain-inactivating mutant form, Myc-SOCS-1R105E,
were kindly provided by Akihiko Yoshimura (Life Sciences Institute,
Kurume, Japan). Monoclonal antibody to phosphotyrosine (4G10) and
polyclonal antibody to Jak2 were purchased from Upstate Biotechnol-
ogy. Polyclonal antibody to phosphorylated Tyr-1007/Tyr-1008 of Jak2
was purchased from Santa Cruz Biotechnology, monoclonal and polyclonal
antibodies to ubiquitin were from Santa Cruz Biotechnology (Santa Cruz,
CA) and Novocastra Laboratories Ltd. (Newcastle, United Kingdom),
respectively. Monoclonal antibodies to Stat5 and SHP-2 were obtained
from BD Transduction Laboratories. Polyclonal antibody to SOCS-1
and monoclonal antibodies to phosphorylated Tyr-694 of Stat5 were
purchased from Zymed Laboratories Inc. Monoclonal antibodies to Myc
were purchased from Santa Cruz Biotechnology and Roche Applied
Science, respectively. Monoclonal antibody (U6) to the PRLR was
provided by Paul Kelly (Paris, France). Phosphothiolated SOCS-1
antisense oligonucleotides were synthesized by Alpha DNA Inc. (Sherbrooke,
Quebec, Canada). Protein A-Sepharose beads were purchas-
ed from Amersham Biosciences. Ovine PRL used for treatment of
was obtained from Sigma.

Ni2 Cell Culture and SOCS-1 Antisense Oligonucleotide Treat-
ments—The Nb2 rat pre-T lymphoma, which is PRL-dependent for
growth, were provided by Peter Gout (BC Cancer Agency, Vancouver,
British Columbia, Canada). These were grown to confluence in RPMI 1640
media containing 10% fetal bovine serum (FBS), 10% horse serum, 7.5%
sodium bicarbonate, 5 mM β-mercaptoethanol, 200 mM glutamine
and then starved for 18 h in starvation media (RPMI 1640 media contain-
ing 10% horse serum, 7.5% sodium bicarbonate, 5 mM β-mercaptoethanol,
200 mM glucose) and stimulated with ovine PRL at 1 μg/mL. For
blocking SOCS-1 expression, phosphothiolated antisense oligonucleo-
tides to SOCS-1 were 5′-ACCTCCGCTGTTGCTGCTGAGAC-3′) were
designed to recognize the initiation site of SOCS-1 protein.
Oligonucleotides were incubated with cells at a concentration of 15 μM
for a 16-h period.

HC11 Cell Culture—HC11, mouse mammary epithelial cells, ob-
tained from Nancy Hynes (Friedrich Miescher Institute, Basel,
Switzerland) and Bernd Groener (Georg Speyer Haus, Frankfurt,
Germany) were grown to confluence in RPMI 1640 media contain-
ing 10% FBS, insulin (5 μg/mL), and epidermal growth factor (10 ng/mL).
Cells were then differentiated to incubating them for 3 days in
RPMI 1640 media containing 10% FBS, insulin (5 μg/mL), and hydro-
cortisone (1 μM). Cells were starved in RPMI 1640 media containing
insulin (5 μg/mL) and hydrocortisone (1 μM) and stimulated by ovine
PRL at 1 μg/mL.

Transient Transfection—The human embryonic 293 cells were grown
in Dulbecco's modified Eagle's medium (4.5 g/liter glucose) contain-
ing 10% FBS. Approximately 5 × 106 cells per plate were plated and then co-
transfected (by calcium phosphate method) with expression plasmids encod-
ing the Nb2 form of the PRLR, PRLRY382F, SHP-2WT, SHP-2CA,
SOCS-1, ubiquitin, SOCS-1R105E, Stat-5, and Jak2 (0.1–1.0 μg). After
18 h of expression, the cells were starved overnight by serum depriva-
tion and then stimulated by PRL at 1 μg/mL for different periods of time
as indicated for each experiment.

Cell Lysis—Cells were lysed in RIPA lysis buffer (50 mM Tris-HCl,
pH 7.4, 1% Nonidet, 0.25% sodium deoxycholate, 150 mM NaCl, 1
mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL (aprotinin,
leupeptin, and pepstatin), 1 mM sodium orthovanadate, 1 mM NaF).
The lysates were cleared by centrifugation at 12,000 × g for 10 min at 4°C
for insoluble material. Protein concentrations were measured
using the Bradford technique.

Immunoprecipitation—Immunoprecipitations were performed on
cellular lysates obtained from Nb2, HC11, or 293 cells that were tran-
siently transfected as indicated for each specific experiment. Immuno-
precipitations were carried out on cell lysates for 3 h at 4°C using
the specified antibodies and protein A-Sepharose beads. Immunoprecipi-
tations were then washed with HNTG buffer (20 mM HEPES, pH 7.5, 150
mM NaCl, 0.1% Triton X-100, 10% glycerol) and separated on SDS-
PAGE.

Western Blotting—Standardized amounts of protein obtained from ei-
ther whole cell lysates or immunoprecipitations were loaded and sepa-
rated on an appropriate concentration of SDS-PAGE. Western blotting
analysis was performed using the specified antibodies for each experi-
ment. Proteins were revealed using chemiluminescence (Lumi-Light Plus,
Roche Diagnostics) following the manufacturer's instructions.

Luciferase Assay—The assay was carried out as described previously
(4). 293 cells were transiently co-transfected with expression vectors as
described for each experiment along with the β-galactosinase promoter/
 luciferase reporter construct (31) and the internal control expression
vector encoding β-galactosidase. Cells were lysed in lysis buffer (1% Triton X-100, 15 mM MgSO4, 4 mM EGTA, 1 mM dithiothreitol) and then
centrifuged at 12,000 × g to clear the lysates. Lysates were mixed with
ATP buffer (1 mM ATP, 15 mM KH2PO4, pH 7.8, 15 mM MgCl2), and
luciferase assay was carried out following the addition of luciferin
substrate (Roche Diagnostic) using the Dynex luminometer (Chantilly,
VA). Relative luciferase units were normalized against β-galactosidase
values, and a representative of five different experiments is presented
as relative light units.

RESULTS

Prolactin Dependent Jak2/SOCS-1 Association Leads to
Ubiquitination and Degradation of Jak2—SOCS-1 protein nega-
tively regulates the signaling of various cytokine receptors. It
was shown to inhibit Jak2 kinase activity through an interac-
tion between the SH2 domain of SOCS-1 and phosphoryl-
ylated Tyr-1007 of Jak2 (24), leading to the proteasomal degradation of
Jak2 (28–30). Similar to other cytokines, the negative-feedback
loop of SOCS-1 in PRL signaling has been previously established as
PRL activation leads to the induction of SOCS-1 mRNA (32), Jak2/SOCS-1 interaction, inhibition of activation of gene transcription (39), and prevent-
ion of lactation (34). We initially intended to determine whether the asso-
ciation of Jak2/SOCS-1 would lead to the ubiquitination and
 degradation of Jak2 kinase in response to PRL stimulation.
The pre-T lymphoma Nb2 cells were grown to confluence,
starved by serum deprivation for 18 h, and then stimulated
with PRL for the time points indicated in Fig. 1A. Cells were
then collected and lysed, immunoprecipitations using a poly-
clonal antibody to Jak2 were carried out, and a sample was
included containing the antibody and lysis buffer as a negative
control. Proteins were separated on SDS-PAGE and trans-
ferred to a nitrocellulose membrane. Initially, Western blotting
analysis using either a monoclonal antibody to phosphoty-
rosine or a polyclonal antibody to phosphorylated Tyr-1007/
Tyr-1008 of Jak2 indicated that indeed PRL induces transient
phosphorylation of Jak2 at tyrosines 1007 and 1008, returning
to basal level by 3 h post-treatment. Western blotting analysis using a polyclonal antibody to Jak2 revealed that
there was a time-dependent decrease in Jak2 protein level
starting at 3 h post-treatment and continued to decrease,
reaching stable low levels of the protein by 6 h (Fig. 1A, middle panel). Blotting with the polyclonal antibody to Jak2 revealed two bands of
which the upper one corresponded to Jak2 observed in the phosphotyrosine and phospho-Jak2 immunoblots.
The nature of the lower band detected by the antibody to Jak2 is not yet clear. To determine whether the decrease in Jak2 protein level is due to ubiquitination, the membrane was stripped and reprobed with a polyclonal antibody to ubiquitin.
As shown in Fig. 1A, lower panel, ubiquitinated Jak2, migrat-
ing as a smear, could be detected starting at 1 h, reaching
a maximum level by 3 h and gradually decreasing thereafter up to
a 24-h time point examined. We also examined PRL-medi-
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ated Jak2 ubiquitination/degradation in the PRL-responsive mouse mammary epithelial HC11 cells and observed that it followed a pattern similar to that seen in Nb2 cells (Fig. 1B).

Together, the data indicate that PRL stimulation leads to a time-dependent ubiquitination and degradation of Jak2.

Using Nb2 cells, we next investigated whether PRL stimulation would lead to an increase in SOCS-1 protein level and, consequently, the interaction between Jak2 and SOCS-1 as SOCS-1 is known to act in a negative-feedback loop. As shown in Fig. 1C, upper panel, PRL stimulation led to a time-dependent increase in SOCS-1 protein level as detected by Western blotting using a polyclonal antibody to SOCS-1. As a positive control for SOCS-1 detection, extracts of mouse thymus were used as described under “Experimental Procedures.” Interestingly, there was a detectable amount of SOCS-1 protein present under basal conditions and the highest level detected was at a 3–6-h stimulation with PRL. SOCS-1 protein level returned to basal levels at 16–24 h following PRL stimulation. In agreement with the increase in SOCS-1 protein, SOCS-1 immunoprecipitations followed by Western blotting using a polyclonal antibody to Jak2 revealed a time-dependent increase in the formation of a Jak2/SOCS-1 complex following PRL stimulation. Jak2/SOCS-1 association was detected at 30 min and peaked at 3–6 h following treatment (Fig. 1C, middle panel). When the membrane was stripped and reprobed with a polyclonal antibody to SOCS-1, it revealed again the time-dependent increase in SOCS-1 protein levels (Fig. 1C, lower panel).

To determine the contribution of PRL-induced SOCS-1 expression in Jak2 degradation, SOCS-1 protein expression was blocked in Nb2 cells using antisense oligonucleotides to SOCS-1 mRNA. Nb2 cells were grown and then starved and either left untreated or treated with the antisense oligonu-
The Carboxyl-terminal Tyrosine of the PRLR Regulates the Kinetics of Jak2 Ubiquitination/Degradation—We have previously reported that the COOH-terminal tyrosine of the PRLR plays a vital role in the regulation of Jak2/Stat-5 signaling pathway leading to gene activation (4) and is a recruitment site for SHP-2 (9). Here we examined initially the role of the COOH-terminal tyrosine of the PRLR in regulating Jak2 stability. For these studies, the human embryonic kidney 293 cells were transiently co-transfected with expression vectors encoding the wild type Nb2 form of the PRLR or the COOH-terminal tyrosine mutant form of the receptor in which tyrosine 382 was mutated to phenylalanine, PRLRY382F, along with expression vectors for Jak2 and SOCS-1. We specifically used the Nb2 form of the PRLR (35) because we have previously shown that this form is biologically active and that the COOH-terminal tyrosine, Tyr-382, is the major tyrosine phosphorylation site in this receptor form (4, 31) and is a recruitment site for SHP-2 (9). Cells were starved for 18 h, stimulated with PRL for 30 min, 1 h, or 3 h, and then collected and lysed. Cellular lysates were prepared for immunodetection with a polyclonal antibody to Jak2. As shown in Fig. 2A, the protein level of Jak2 remained relatively constant over the 3-h time course of PRL stimulation in samples overexpressing the Nb2 wild type receptor. In contrast, in samples overexpressing the COOH-terminal tyrosine mutant form of the receptor, there was a rapid decrease in the protein level of Jak2 starting at 30 min and clearly visible at 1 h post-treatment. After 3 h of PRL stimulation, there was little detectable Jak2 present. Therefore, the COOH-terminal tyrosine of the PRLR plays an important role in Jak2 stability. We next sought to determine whether protein degradation was specific to Jak2, and for that reason, 293 cells were transiently co-transfected with expression vectors encoding either the Nb2 PRLR or Nb2 PRLRY382F mutant along with expression vectors for SOCS-1 and Stat5. As shown in Fig. 2A, lower panel, unlike the pattern for Jak2 degradation, Stat5

using a monoclonal antibody to Stat5 (lower panel). B, cellular lysates obtained from 293 cells that were transiently co-transfected with expression vectors for the Nb2PRLRY382F (YF) along with SOCS-1. 24 h following transfection, cells were starved for an overnight period and then stimulated with PRL, for the times indicated. Whole cell lysates were then immunoblotted with a polyclonal antibody to Jak2 (upper panel). A similar transfection was carried out as above, but an expression plasmid encoding Stat5 was included. Following starvation and stimulation with PRL, cells were lysed and whole cell lysates were immunodetected to SOCS-1 for an overnight period. As shown in Fig. 1D, the presence of the antisense oligonucleotides prevented the increase in SOCS-1 expression at all of the time points examined. The effect was particularly obvious at the 6-h time point. This correlated with an increase in the stability of Jak2 observed at the different time points in samples pretreated with the antisense oligonucleotides (Fig. 1D, lower panel). At the 6-h time point, the attenuation in degradation of Jak2 was most apparent. Thus, blocking SOCS-1 protein expression impedes the time-dependent degradation of Jak2. Taken together, PRLR stimulation induces a time-dependent increase in SOCS-1 expression accompanied by an increase in the association of Jak2/SOCS-1, leading ultimately to a time-dependent ubiquitination and degradation of Jak2.

The COOH-terminal tyrosine of the PRLR regulates ubiquitination/degradation of Jak2 and Jak2/ SOCS-1 association. A, 293 cells were transiently co-transfected with the exception of the control sample (Ctl) with expression vectors for the Nb2 form of the PRLR (WT) or the corresponding COOH-terminal tyrosine mutant form PRLRY382F (YF) along with SOCS-1. 24 h following transfection, cells were starved for an overnight period and then stimulated with PRL, for the times indicated. Whole cell lysates were then immunoblotted with a polyclonal antibody to Jak2 (upper panel). A similar transfection was carried out as above, but an expression plasmid encoding Stat5 was included. Following starvation and stimulation with PRL, cells were lysed and whole cell lysates were immunodetected to SOCS-1 for an overnight period. As shown in Fig. 1D, the presence of the antisense oligonucleotides prevented the increase in SOCS-1 expression at all of the time points examined. The effect was particularly obvious at the 6-h time point. This correlated with an increase in the stability of Jak2 observed at the different time points in samples pretreated with the antisense oligonucleotides (Fig. 1D, lower panel). At the 6-h time point, the attenuation in degradation of Jak2 was most apparent. Thus, blocking SOCS-1 protein expression impedes the time-dependent degradation of Jak2. Taken together, PRLR stimulation induces a time-dependent increase in SOCS-1 expression accompanied by an increase in the association of Jak2/SOCS-1, leading ultimately to a time-dependent ubiquitination and degradation of Jak2.

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The Carboxyl-terminal Tyrosine of the PRLR Regulates the Kinetics of Jak2 Ubiquitination/Degradation—We have previously reported that the COOH-terminal tyrosine of the PRLR plays a vital role in the regulation of Jak2/Stat-5 signaling pathway leading to gene activation (4) and is a recruitment site for SHP-2 (9). Here we examined initially the role of the COOH-terminal tyrosine of the PRLR in regulating Jak2 stability. For these studies, the human embryonic kidney 293 cells were transiently co-transfected with expression vectors encoding the wild type Nb2 form of the PRLR or the COOH-terminal tyrosine mutant form of the receptor in which tyrosine 382 was mutated to phenylalanine, PRLRY382F, along with expression vectors for Jak2 and SOCS-1. We specifically used the Nb2 form of the PRLR (35) because we have previously shown that this form is biologically active and that the COOH-terminal tyrosine, Tyr-382, is the major tyrosine phosphorylation site in this receptor form (4, 31) and is a recruitment site for SHP-2 (9). Cells were starved for 18 h, stimulated with PRL for 30 min, 1 h, or 3 h, and then collected and lysed. Cellular lysates were prepared for immunodetection with a polyclonal antibody to Jak2. As shown in Fig. 2A, the protein level of Jak2 remained relatively constant over the 3-h time course of PRL stimulation in samples overexpressing the Nb2 wild type receptor. In contrast, in samples overexpressing the COOH-terminal tyrosine mutant form of the receptor, there was a rapid decrease in the protein level of Jak2 starting at 30 min and clearly visible at 1 h post-treatment. After 3 h of PRL stimulation, there was little detectable Jak2 present. Therefore, the COOH-terminal tyrosine of the PRLR plays an important role in Jak2 stability. We next sought to determine whether protein degradation was specific to Jak2, and for that reason, 293 cells were transiently co-transfected with expression vectors encoding either the Nb2 PRLR or Nb2 PRLRY382F mutant along with expression vectors for SOCS-1 and Stat5. As shown in Fig. 2A, lower panel, unlike the pattern for Jak2 degradation, Stat5
protein level did not change over the time course of PRL stimulation in the different samples. Therefore, Stat5 does not undergo a similar protein degradation pattern as that observed for Jak2. Together, the results indicate that the COOH-terminal tyrosine of the PRLR regulates the stability of Jak2 kinase in response to PRL stimulation.

We next examined whether the COOH-terminal tyrosine of the PRLR regulates the interaction between Jak2 and SOCS-1, leading to the observed differential stability of Jak2. Cells transiently co-transfected as noted above were starved for 18 h and stimulated with PRL for 10 or 30 min. These time points of stimulation were selected because there was minimal degradation of Jak2. Immunoprecipitations were carried out using a polyclonal antibody to Jak2, and immune complexes were blotted with a monoclonal antibody to Myc, recognizing Myc-tagged SOCS-1 protein (Fig. 2B). As expected, PRL stimulation led to Jak2/SOCS-1 interaction in all of the samples. Interestingly, more SOCS-1 protein was found to co-immunoprecipitate with Jak2 in samples overexpressing the PRLRY382F compared with samples overexpressing wild type PRLR. Reprobing the membrane with a polyclonal antibody to Jak2 showed equal immunoprecipitations of Jak2 (Fig. 2B, lower panel). These data indicate that a mechanism(s) operating downstream of the COOH-terminal tyrosine of the PRLR regulates Jak2 and SOCS-1 to form a complex in response to PRL stimulation.

Because SOCS-1-mediated degradation of associated proteins occurs through ubiquitination, we next examined the role of the COOH-terminal tyrosine of the PRLR in Jak2 ubiquitination. 293 cells were transiently co-transfected with expression vectors for either the wild type Nb2 form of the PRLR or the COOH-terminal tyrosine mutant of the Nb2 form, PRLRY382F, along with expression vector for SOCS-1. Cells were starved and stimulated with PRL at different time points, and immunoprecipitations were carried out using a polyclonal antibody to Jak2. Immunoprecipitates were blotted with a polyclonal antibody to ubiquitin (Fig. 2C). As can be observed, PRLR activation led to Jak2 ubiquitination. Interestingly, the rate of Jak2 ubiquitination was faster, reaching a maximal level within 1 h of stimulation in samples overexpressing the COOH-terminal tyrosine mutant form of the PRLR compared with samples overexpressing the wild type PRLR where Jak2 ubiquitination became apparent only following 3 h of stimulation. Reprobing the membrane with a polyclonal antibody to Jak2 showed a similar pattern of Jak2 degradation to that observed in Fig. 2A. Because SOCS-1 forms a complex with Jak2, we next examined whether the COOH-terminal tyrosine of the PRLR would regulate the stability of SOCS-1 protein. As can be seen in Fig. 2D, the protein level of SOCS-1 decreases with time in the presence of PRLRY382F compared with PRL-RWT. Thus, the COOH-terminal tyrosine of the PRLR regulates the kinetics of Jak2 and SOCS-1 association and ultimately Jak2/SOCS-1 ubiquitination and degradation.

SHP-2 Modulates Formation of Jak2/SOCS-1 Complex and Protein Stability of Jak2—We have previously reported that the COOH-terminal tyrosine of the PRLR is a site for SHP-2 recruitment (9). Therefore, we sought to determine whether the regulation of Jak2 ubiquitination and degradation by the COOH-terminal tyrosine of the PRLR is because of the function of SHP-2. 293 cells were transiently co-transfected with expression vectors for either the wild type SHP-2 (SHP-2WT) or a catalytically inactive mutant of SHP-2 where cysteine 463 is mutated to alanine (SHP-2CA) along with expression vectors for the Nb2 form of the PRLR and SOCS-1 (Fig. 3A). Cells were starved overnight and stimulated with PRL for 10 min, 30 min, or 1 h. Immunoprecipitations were carried out using a polyclonal antibody to Jak2. Immunocomplexes were prepared for

Fig. 3. The catalytic activity of SHP-2 is involved in regulating Jak2 and SOCS-1 degradation by modulating Jak2/SOCS-1 interaction. A, using a polyclonal antibody to Jak2, immunoprecipitations (IP) of Jak2 were carried out using cell lysates from 293 cells transiently co-transfected (with expression vectors for either wild type SHP-2 or SHP-2CA along with the Nb2 form of the PRLR and SOCS-1), starved, and stimulated with PRL for the time points indicated. Immunocomplexes were separated on SDS-PAGE and then immunodetected using a monoclonal antibody to Myc-tag detecting SOCS-1 (upper panel) or a polyclonal antibody to Jak2 (lower panel). A negative control sample (Ctl) was included containing protein A-Sepharose beads, the antibody to Jak2, and lysis buffer. B, 293 cells were transiently co-transfected as described in A, starved, and stimulated with PRL for the time points indicated. Cells were lysed, and immunoprecipitations (IP) of Jak2 were performed. Immunoblotting with polyclonal antibody to ubiquitin was performed (upper panel). Total cell lysates were immunodetected using a polyclonal antibody to Jak2 (lower panel). C, 293 cells transiently co-transfected as described in A, starved, and stimulated with PRL for the indicated time points were used for immunoprecipitations of SOCS-1 using a polyclonal antibody to SOCS-1. Immunocomplexes and total cell lysates were immunodetected using a monoclonal antibody to Myc tag recognizing SOCS-1 (upper panel) or a monoclonal antibody to SHP-2 (lower panel). Ubiq., ubiquitination.
immunodetection with a monoclonal antibody to the Myc tag, which recognizes SOCS-1 (Fig. 3A, upper panel). As can be observed, SOCS-1 protein co-immunoprecipitated with Jak2 following PRL stimulation in the different samples. However, in samples overexpressing SHP-2CA, a notably higher association between Jak2 and SOCS-1 was observed at all of the time points compared with samples overexpressing SHP-2 wild type. When the membrane was probed with a polyclonal antibody to Jak2, similar levels were seen (Fig. 3A, lower panel). Also equivalent levels of SHP-2WT and SHP-2CA were observed in the different samples (data not shown). These data indicate that SHP-2 regulates the formation of a Jak2/SOCS-1 complex downstream of the PRLR.

The association between Jak2 and SOCS-1 leads to the ubiquitination and degradation of Jak2. We next sought to determine whether the increased association between Jak2 and SOCS-1 observed in the presence of SHP-2CA would translate into a faster rate of ubiquitination and degradation of Jak2. For that reason, we transiently co-transfected 293 cells with expression vectors for either SHP-2WT or SHP-2CA along with expression vectors for the Nb2 form of the PRLR and SOCS-1. Cells were starved overnight and stimulated with PRL for 30 min, 1 h, or 3 h. Cell lysates were used for immunoprecipitations of Jak2 using a polyclonal antibody to Jak2 and immunoblotted with a polyclonal antibody to ubiquitin (Fig. 3B). As can be seen, following PRL stimulation, the rate of Jak2 ubiquitination occurred faster in samples overexpressing SHP-2CA, peaking at 1 h following stimulation compared with samples overexpressing SHP-2WT where elevated levels of ubiquitination was observed following 3–5 h of stimulation. To examine whether the increased rate of ubiquitination of Jak2 was associated with a decrease in the amount of Jak2, whole cell lysates from the above transfection were separated on SDS-PAGE and immunodetected with a polyclonal antibody to Jak2 (Fig. 3B, upper panel). Following PRL stimulation, there was an accelerated decrease in the amount of Jak2 in samples overexpressing SHP-2CA compared with samples overexpressing SHP-2WT. Therefore, the phosphatase SHP-2 regulates Jak2/SOCS-1 interaction hence controls the processes of ubiquitination and degradation of Jak2.

The association between SOCS-1 and Jak2 results in the formation of a complex targeted for degradation. We next investigated whether SHP-2 function leads to modulation in SOCS-1 protein degradation in a manner similar to the changes observed for Jak2 protein. We obtained cellular lysates from 293 cells that were co-transfected with expression vectors for either SHP-2WT or SHP-2CA along with expression vectors for the Nb2 form of the PRLR and SOCS-1. As shown in Fig. 3C, immunoprecipitations of SOCS-1 followed by Western blotting with a monoclonal antibody to the Myc tag (recognizing SOCS-1) indicate that following PRL stimulation there is a time-dependent loss in the amount of total SOCS-1 protein in samples overexpressing SHP-2CA compared with samples overexpressing SHP-2WT (Fig. 3C, upper panel). This indicates that inactivation of SHP-2 leads to the degradation of SOCS-1 protein concurrently with the degradation of Jak2. Reprobing the membrane with a monoclonal antibody to SHP-2 revealed equivalent overexpression of SHP-2 and SHP-2CA (Fig. 3C, lower panel).

To further confirm the role of SHP-2 in regulating the ubiquitination/degradation of Jak2, we co-overexpressed in 293 cells Jak2 along with PRLR Nb2, SOCS-1, SHP-2WT, or SHP-2CA. As can be seen in Fig. 4, in the presence of SHP-2WT, ubiquitination of Jak2 reached a maximum at 1 h following stimulation by PRL. However, the rate of ubiquitination of Jak2 occurred at a faster rate in the presence of SHP-2CA in which this process started before ligand stimulation. Reprobing the membrane with a polyclonal antibody to Jak2 indicated that degradation of Jak2 was observed to correlate with its ubiquitination pattern as Jak2 protein levels were maintained longer in the presence of SHP-2WT compared with SHP-2CA (Fig. 4). Furthermore, immunoblotting of whole cell lysates with monoclonal antibodies to Myc tag (recognizes SOCS-1) and SHP-2 revealed a gradual degradation of SOCS-1 and SHP-2CA proteins but not SHP-2WT. Equal protein samples were loaded as confirmed by reprobing the membrane with a monoclonal antibody to β-tubulin (lower panels). Ubiquitination.

Fig. 4. Degradation of Jak2/SOCS-1 is regulated by the catalytic domain of SHP-2. 293 cells were transiently co-transfected as in Fig. 3 with the addition of Jak2 cDNA. Cells were starved and stimulated by PRL for the time points indicated. Cell lysates were immunoprecipitated (IP) for Jak2 and separated on SDS-PAGE. Immunoblot with a monoclonal antibody to ubiquitin (upper panel) and reprobing for Jak2 were carried out. Whole cell lysates were probed with monoclonal antibodies to SHP-2, Myc, and β-tubulin (lower panels). Ubiquitination.
transfected with expression vectors for the Nb2 form of the PRLR either in the absence or the presence of overexpression of SHP-2. Cells were starved by serum deprivation and then whole cell lysates were separated on SDS-PAGE and immunodetected with a polyclonal antibody to phosphorylated Tyr-1007/Tyr-1008 of Jak2. As can be seen in Fig. 5B, Jak2 phosphorylation was observed upon PRL stimulation in all of the samples; however, the level of phosphorylation was clearly higher in samples overexpressing SHP-2CA. The membrane was reblotted with a polyclonal antibody to Jak2 and then with a monoclonal antibody to SHP-2 to confirm similar levels of protein expression in all of the samples. These results further confirm that Tyr-1007 of Jak2 is a substrate for SHP-2.

Because dephosphorylation of Tyr-1007 of Jak2 by different phosphatases such as CD45 (13) and PTP1B (14–17) have been linked to negative regulation of the kinase, we next examined the role of dephosphorylation of Jak2 by SHP-2 on the tyrosine phosphorylation of Stat5 as an indicator of the signaling activity of Jak2 downstream of the PRLR. 293 cells were transiently co-transfected with expression vectors for either SHP-2WT or SHP-2CA along with expression vectors for Stat5. Cells were starved and then stimulated with PRL for 10 or 30 min. Whole cell lysates were separated on SDS-PAGE and immunodetected with a monoclonal antibody to phospho-Tyr-694 of Stat5. As can be observed in Fig. 5C, following PRL stimulation, tyrosine phosphorylation of Stat5 was only detected in samples overexpressing SHP-2WT but not in samples overexpressing SHP-2CA. These data confirm previous studies indicating that SHP-2 is required for PRLR signaling (8–10, 36) and correlate the dephosphorylation at Tyr-1007/Tyr-1008 of Jak2 by SHP-2 leading to the activation of Jak2. Taken together, the phosphatase SHP-2 dephosphorylates Jak2 on Tyr-1007/Tyr-1008, encompassing the recruitment site for SOCS-1, and this dephosphorylation process plays a positive role in the regulation of Jak2 kinase activity.

**SHP-2 Regulates SOCS-1-mediated Inhibition of PRLR Signaling**—Activation of the PRLR leads to the induction of gene expression of various milk proteins. Measuring gene promoter activation of the milk protein β-casein by employing luciferase assays is used as a marker of PRLR activation of signaling. To investigate SHP-2-mediated regulation of Jak2/SOCS-1 interaction in PRLR signaling to gene activation, we co-transfected 293 cells with expression plasmids encoding either SHP-2WT or SHP-2CA along with the Nb2 form of the PRLR, Stat5, SOCS-1, β-galactosidase, and the reporter construct β-casein/lux. Following transfection, cells were starved and stimulated with PRL overnight and lysed and luciferase assays were performed. As shown in Fig. 6A (a representative experiment), PRL stimulation led to the activation of the β-casein gene promoter (15-fold). As expected, overexpressing SOCS-1 potently inhibited this activation. Interestingly, overexpressing SHP-2 led to the recovery of PRL signals (~80%). In contrast, PRLR signaling was not recovered in the presence of SHP-2CA. This indicates that the catalytic activity of SHP-2 attenuates
FIG. 6. SHP-2 recovers SOCS-1-mediated inhibition of PRLR signaling. A, 293 cells were co-transfected with expression vectors for the Nb2 form of the PRLR, Stat5, β-galactosidase, β-casein-lux reporter construct, and a combination of SOCS-1, SHP-2, or SHP-2CA as indicated. 24 h following transfection, cells were starved and stimulated by PRL for an overnight period and prepared for luciferase assay. One representative experiment is shown, and the data are presented as relative light units (left panel). Whole cell lysates obtained from 293 cells that were transiently co-transfected with expression vectors for the Nb2 form of the PRLR, Jak2, Stat5, and a combination of SOCS-1, SHP-2, or SHP-2CA as indicated were immunoblotted with a monoclonal antibody to phospho-Tyr-694 of Stat5. The membrane was stripped then reprobed with a monoclonal antibody to Stat5 (right panel). B, 293 cells that were co-transfected with expression vectors for the Nb2 form of the PRLR, Stat5, β-galactosidase, β-casein-lux reporter construct, and a combination of SOCS-1, the SH2 domain-inactivating mutant of SOCS-1, SOCS-1R105E, or the catalytically inactive SHP-2 mutant form, SHP-2CA, as indicated. 24 h following transfection, cells were starved and stimulated by PRL for an overnight period and prepared for luciferase assay. Data presented as relative light units and a representative experiment are presented (left panel). Whole cell lysates obtained from 293 cells that were transiently co-transfected with expression vectors for the Nb2 form of the PRLR, Jak2, Stat5, and a
SOCS-1-mediated inhibition of PRLR signaling to milk protein gene promoter activation. As a second measure of signaling activity of the PRLR, we examined the tyrosine phosphorylation level of Stat5. 293 cells transiently co-transfected with expression vectors for SHP-2WT or SHP-2CA along with the Nb2 form of the PRLR, SOCS-1 and Stat5. Cells were then starved and stimulated with PRL for 10 min. Cell lysates were separated on SDS-PAGE and prepared for Western blotting with a monoclonal antibody to phospho-Tyr-694 of Stat5. As shown in Fig. 6, _upper right panel_, PRL stimulation led to tyrosine phosphorylation of Stat5, which was inhibited by overexpressing SOCS-1. However, tyrosine phosphorylation of Stat5 was clearly restored in samples overexpressing SHP-2WT but not in samples overexpressing SHP-2CA. Therefore, the phosphatase SHP-2 through its catalytic activity is able to block the inhibitory effects of SOCS-1 in PRLR signaling, leading to Stat5 phosphorylation and gene promoter activation.

To further confirm that SHP-2 contribution to PRLR signaling is mediated through the regulation of Jak2/SOCS-1 interaction, we used an SH2 domain-inactivating mutant form of SOCS-1, SOCS-1R105E (29), which blocks the recruitment of SOCS-1 to Jak2 in the functional luciferase assay system. We co-transfected 293 cells with expression plasmids encoding either SOCS-1 or SOCS-1R105E along with the Nb2 form of the PRLR, Stat5, SHP-2CA, β-galactosidase, and the reporter construct β-casein/lux. Cells were starved and stimulated with PRL overnight and lysed, and luciferase assay was performed. As shown in Fig. 6B (_a representative experiment_), the data revealed that stimulation with PRL led to the activation of the β-casein gene promoter and as expected overexpression of SOCS-1 blocked this effect. Furthermore, overexpression of SOCS-1R105E had no effect on PRL-mediated gene promoter activation, indicating that SOCS-1-mediated inhibition of PRLR signaling requires its SH2 domain as has been reported for other cytokine receptor systems (29). Co-overexpressing SHP-2CA mutant with SOCS-1 as expected inhibited PRLR signaling. Significantly, co-overexpressing SHP-2CA with SOCS-1R105E restored luciferase activity, indicating that the ability of SHP-2CA to inhibit PRLR signaling requires a functional SH2 domain of SOCS-1. Together, the data indicate that the positive effect displayed by SHP-2 in PRLR signaling is because of the inhibition of Jak2 association with SOCS-1.

We next investigated whether blocking SOCS-1 recruitment to Jak2 would modulate the inhibitory effects of SHP-2CA on Stat5 tyrosine phosphorylation. 293 cells were transiently co-transfected with expression vectors for the Nb2 form of the PRLR, SHP-2CA, with either SOCS-1 or SOCS-1R105E as indicated in Fig. 6B, _right panel_. Cell lysates were separated on SDS-PAGE, transferred to a nitrocellulose membrane, and then blotted with antibodies to phosphorylated Tyr-694 of Stat5. As expected, tyrosine phosphorylation of Stat5 was induced by PRL and inhibited by SOCS-1 overexpression. Inhibition of Stat5 tyrosine phosphorylation was not observed in samples overexpressing SOCS-1R105E, indicating the essential role of the SH2 domain of SOCS-1 in mediating the inhibitory effects of SOCS-1. Moreover, overexpressing SHP-2CA along with SOCS-1 as expected blocked PRL-mediated Stat5 phosphorylation. In contrast, co-overexpressing SHP-2CA together with SOCS-1R105E relieved the inhibitory effects of SHP-2CA on PRL-mediated Stat5 tyrosine phosphorylation (_Fig. 6B, right panel_). These results taken together indicate that SHP-2 modulates the signaling activity of the PRLR by regulating the interaction of Jak2/SOCS-1, thereby blocking the inhibitory effects of SOCS-1.

The capability of SOCS-1R105E to restore tyrosine phosphorylation of Stat5 and β-casein gene promoter activation downstream of the PRLR led us to examine the role of SOCS-1R105E in influencing the stability of Jak2. As shown in Fig. 6C, more Jak2 was observed in samples overexpressing SOCS-1R105E compared with samples overexpressing SOCS-1 wild type in the presence of either SHP-2WT or SHP-2CA. Interestingly, SOCS-1R105E appears also to be more stable compared with SOCS-1 wild type, suggesting that the degradation SOCS-1 requires a functional SH2 domain. Furthermore, we noticed that a shift in the molecular weight of SOCS-1R105E relative to SOCS-1 wild type could be due to change in charge2 as can be seen in the immunoblot using a polyclonal antibody to SOCS-1. Immunoblotting with a monoclonal antibody to β-tubulin revealed equal protein loading. Therefore, the phosphatase SHP-2 is crucial for PRLR signaling by regulating the interaction of Jak2/SOCS-1 and hence Jak2 degradation.
Strict regulation of intracellular signaling is critical for obtaining accurate biological and physiological responses. Both protein kinases and phosphatases are part of an elaborate network of pathways that control the outcome of cellular signaling activities. It is clear that kinases and phosphatases act to regulate and fine-tune each others’ activities in dynamic temporal and spatial dependent mechanisms.

The protein tyrosine phosphatase SHP-2 has emerged as an important physiological regulator of signaling of a wide variety of cytokines and growth factors. However, the molecular mechanism of action and target(s) of SHP-2, particularly related to cytokine receptor signaling, are not well characterized. Here we report a new mechanism of action for SHP-2 in regulating cytokine receptor signaling, which leads to signal propagation. We determined that the protein tyrosine phosphatase SHP-2 plays an essential role in regulating PRLR signaling by dephosphorylating Tyr-1007 within the activation loop of Jak2, a known recruitment site for SOCS-1, thereby contributing to the protein stability of Jak2 as well as its signaling capacity.

Our results indicate that blocking SHP-2 function downstream of the PRLR by utilizing the COOH-terminal tyrosine mutant form of the PRLR, lacking the association site for SHP-2, as well as SHP-2CA leads to an increased rate of ubiquitination and degradation of Jak2. Significantly, we determined that these events are regulated by SHP-2 through modulating the phosphorylation state of Tyr-1007 of Jak2, influencing the interaction of Jak2/SOCS-1. Moreover, our results also indicate that loss of SHP-2 function leads to an increase in the degradation of SOCS-1 proteins, suggesting that SOCS-1 binding to Jak2 will target PRLR/Jak2/SOCS-1 as a complex to the proteasomal degradation machinery associated with SOCS-1. We are presently investigating the role of SHP-2 in PRLR signaling using a more in vivo approach by developing mammary epithelial cell lines in which the expression of various mutant forms of SHP-2 are controlled.

Phosphorylation of Tyr-1007 of Jak2 is a requirement for the activity of the kinase (18). Our results indicate that following PRL stimulation there is an increase in the phosphorylation of Tyr-1007 of Jak2. Interestingly however, we observed that the same tyrosine is also a target for dephosphorylation by SHP-2. In fact, we observed that less phosphorylation of Tyr-1007 of Jak2 is linked to a higher signaling capacity of the PRLR as the same tyrosine is also a target for dephosphorylation by SHP-2 and activation of the b-casein gene promoter. Furthermore, the fact that the inhibitory effects of SHP-2CA in PRLR signaling were blocked in the presence SOCS-1R105E mutant, unable to bind Jak2, indicates that blocking the interaction of Jak2/SOCS-1 is a major mechanism by which SHP-2 regulates PRLR signaling. Together, our results indicate that dephosphorylation of Tyr-1007 of Jak2 by SHP-2 is a necessary event for PRLR signal propagation.

The seemingly paradoxical role of phosphorylation of Tyr-1007 of Jak2 could be clarified by adopting a dynamic vision of the signaling process as depicted in Fig. 7. Following activation of cytokine receptors, if Jak2 phosphorylation on Tyr-1007 was to be maintained, this would create a recruitment site for the increasingly accumulating inhibitory SOCS-1 protein and would ultimately lead to the degradation of Jak2 and termination of the signal. However, in the presence of the phosphatase SHP-2, Tyr-1007 of Jak2 is dephosphorylated preventing SOCS-1/Jak2 from interacting with one another, impeding ubiquitination/degradation of Jak2 and consequently maintaining the signaling capacity of the receptor/Jak2 complex. The dephosphorylated Jak2 is then rephosphorylated and reactivated by auto/oligomerization. It is conceivable that the cycle of phosphorylation/dephosphorylation of Jak2 could repeat itself for a number of times. The cycle is terminated when SOCS-1 levels are elevated to a certain threshold where the equilibrium is shifted toward degradation of Jak2 rather than dephosphorylation by SHP-2 and reactivation. Intriguingly, immunoblotting with SOCS-1 antibodies performed in Nb2 cells (Fig. 1C) and HC11 cells (data not shown) revealed the presence of high basal amounts of SOCS-1 prior to PRL stimulation, suggesting that the process of phosphorylation/dephosphorylation of Jak2 starts early following PRLR activation. Our results implicate the duration of phosphorylation of Tyr-1007 of Jak2 as a critical determinant in the final outcome of signaling following cytokine receptor activation.

The transmembrane phosphatase CD45 and the cytoplasmic phosphatase PTP1B were both shown to dephosphorylate Tyr-1007 of Jak2. However, these two phosphatases differ from SHP-2 in that they negatively regulate cytokine signaling (13, 15, 16). The reason for the divergence in the function of SHP-2 compared with CD45 and PTP1B is not yet known. It is possible to speculate that the differences might be due to local concentrations of these phosphatases at the receptor/Jak2 complex. Alternatively, it may be explained by the possibility that CD45 and PTP1B possess higher catalytic activities compared with SHP-2. In such a case, Jak2 would be in a continuous dephosphorylated state, which would lead to its permanent inhibition. Further investigation is required to clarify these mechanisms.

This study provides the first example of a protein tyrosine phosphatase that acts in a positive manner via maintaining the signaling capacity of Jak2 through dephosphorylation of Tyr-1007 of the kinase. Possibilities exist in which other phosphatases may act in a similar manner or in which SHP-2 could modulate other kinases in a mode similar to the regulation of Jak2. Interestingly, our data presented here is in agreement with recently published mathematical models employed to describe signal transduction events (37). It was concluded that signal amplitude is controlled primarily by kinases, whereas phosphatases have a more profound effect than kinases on the rate and duration of signaling.

In this report, we demonstrated that the protein tyrosine phosphatase SHP-2 plays a positive role in signaling through dephosphorylating the kinase Jak2 as well as the PRLR. This function of SHP-2 allows signal generation and maintenance by preventing the association of Jak2 to the inhibitory protein SOCS-1. Further exploration should reveal that this function of SHP-2 is not unique to the PRLR signaling system but rather a general mechanism of regulation of Jak2 by SHP-2.

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