Mechanism of protein tyrosine phosphatase 1B-mediated inhibition of leptin signalling

I K Lund1, J A Hansen1, H S Andersen2, N P H Møller1 and N Billestrup1,3

1Signal Transduction, Novo Nordisk A/S, DK-2880 Bagsværd, Denmark
2MedChem Research II, Novo Nordisk A/S, DK-2760 Måløv, Denmark
3Steno Diabetes Center, Niels Steensens Vej 6, DK-2820 Gentofte, Denmark

(Requests for offprints should be addressed to N Billestrup; Email: nbil@steno.dk)

Abstract

Upon leptin binding, the leptin receptor is activated, leading to stimulation of the JAK/STAT signal transduction cascade. The transient character of the tyrosine phosphorylation of JAK2 and STAT3 suggests the involvement of protein tyrosine phosphatases (PTPs) as negative regulators of this signalling pathway. Specifically, recent evidence has suggested that PTP1B might be a key regulator of leptin signalling, based on the resistance to diet-induced obesity and increased leptin signalling observed in PTP1B-deficient mice. The present study was undertaken to investigate the mechanism by which PTP1B mediates the cessation of the leptin signal transduction. Leptin-induced activation of a STAT3 responsive reporter was dose-dependently inhibited by co-transfection with PTP1B. No inhibition was observed when a catalytically inactive mutant of PTP1B was used or when other PTPs were co-transfected. PTP1B was able to dephosphorylate activated JAK2 and STAT3 in vitro, whereas either no or a minimal effect was observed with cluster of differentiation 45 (CD45), PTPα/afii9825 and leukocyte antigen-related (LAR). By utilisation of a selective PTP1B inhibitor, the leptin-induced STAT3 activation was enhanced in cells. In conclusion, these results suggested that the negative regulatory role of PTP1B on leptin signalling is mediated through a direct and selective dephosphorylation of the two signalling molecules, JAK2 and STAT3.

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Introduction

Leptin, the 16 kDa protein product of the obese gene (Zhang et al. 1994), is an adipocyte-derived hormone, secreted in proportion to the total body fat mass (Banks 2003). The primary site of leptin action is the hypothalamus, through which it exerts its key regulatory role by controlling the overall energy homeostasis, i.e. induction of reduced food intake and promotion of weight loss (Ahima & Flier 2000, Korner & Aronne 2003). Defective leptin signalling due to either leptin deficiency or dysfunctional leptin receptors leads to early onset of severe obesity in both humans and rodents (Clement et al. 1998, Ahima & Flier 2000). In human obesity and type 2 diabetes, circulating levels of leptin are usually elevated and consequently accompanied by a reduced sensitivity to leptin (often referred to as leptin resistance) (El Haschimi et al. 2000). At present, the exact molecular mechanism eliciting this physiological insensitivity has not been characterised. However, the existence of impaired leptin transport across the blood–brain barrier and/or the deregulation of the activity of proteins participating in the negative feedback mechanism on leptin signalling seem to constitute important aspects for the presence of leptin resistance (Bjørbaek et al. 1999, El Haschimi et al. 2000, Banks 2003).

Structurally, the leptin receptor belongs to the class I cytokine receptor family (Tartaglia et al. 1995, Tartaglia 1997), for which a common feature is the highly conserved Box 1 and Box 2 motifs, positioned in the cytoplasmic region and known to be of importance for association and activation of signalling molecules (Ihle & Kerr 1995, Kloek et al. 2002). Upon leptin stimulation, the ligand-induced conformational changes of the leptin-bound receptor dimers lead to recruitment of JAK2 proteins to the cytoplasmic domain of the receptor (Tartaglia 1997, Ahima & Flier 2000, Sweeney 2002). Subsequent tyrosine phosphorylation and activation of JAK2 and the leptin receptor result in the creation of docking sites for signalling molecules, such as the STAT3 proteins. In particular, the phosphorylated STAT3 proteins dimerise, translocate into the nucleus and regulate expression of target genes through DNA binding (Ahima & Flier 2000, Levy & Darnell 2002).

The mechanisms controlling and terminating the leptin signal transduction seem more elusive but are believed to include (i) internalisation and degradation of the leptin receptor-ligand complex (possibly via the ubiquitin-proteasome pathway) (Haspel et al. 1996), (ii) feedback inhibition by negative regulators such as the cytokolic suppressors of cytokine signalling (SOCS) (Bjørbaek et al. 1999, Gadina et al. 2001) and the nuclear

The transient character of the leptin-induced tyrosine phosphorylation of JAK and STAT proteins suggests the presence of PTPs, although little is known about the role of these enzymes in the cessation of the leptin signalling pathway. Two elegant studies have recently provided evidence for the presence of an increased insulin sensitivity, resistance to diet-induced obesity and increased energy expenditure in PTP1B-deficient mice (Elchebly et al. 1999, Klaman et al. 2000). The increased insulin sensitivity and prolonged tyrosine phosphorylation of the insulin receptor tyrosine kinase (IRTK) in these mice suggest a direct negative regulatory role of PTP1B on the IRTK, and thus on glucose homeostasis; a notion corroborated by the improved glucose tolerance observed in mice deficient in PTP1B and insulin receptor substrate-2 (IRS-2) (Kushner et al. 2004) and in rodents treated with PTP1B antisense oligonucleotides (Rondinone et al. 2002, Zinker et al. 2002, Gum et al. 2003). The resistance to diet-induced obesity and increased energy expenditure, together with a recent study suggesting JAK2 as a substrate of PTP1B (Myers et al. 2001), clearly points to a central role of PTP1B in leptin signalling. In agreement, subsequent studies have illustrated augmented leptin signalling in the two strains of PTP1B knockout mice (Cheng et al. 2002, Zabolotny et al. 2002). Recent reports have additionally provided support for a negative regulatory role of PTP1B in JAK/STAT-mediated signalling upon induction with various cytokines such as leptin (Kaszubska et al. 2002), prolactin (Aoki & Matsuda 2000) and growth hormone (Gu et al. 2003).

In order to understand the molecular mechanism behind the action of PTP1B on the signalling molecules transmitting the effects of leptin, we have investigated the role of PTP1B and related PTPs on leptin signalling using (i) a leptin-responsive STAT3-dependent reporter assay, (ii) an in vitro dephosphorylation assay of JAK2 and STAT3 and (iii) a prodrug inhibitor with selectivity for PTP1B applied to cells in which STAT3 DNA binding were measured.

Materials and methods

Materials

All cell culture reagents were purchased from GIBCO-BRL Life Technologies (Gaithersburg, MD, USA) and Sigma unless otherwise is noted. Other chemicals were analytic grade from Merck, Darmstadt, Germany. Water was purified in a Millipore purification system (18 Megohm/cm; Millipore Inc., Glostrup, Denmark). Insulin and leptin were from Novo Nordisk A/S, (Bagsvaerd, Denmark).

Cell culture

Human embryonic kidney (HEK) 293 cells stably transfeceted with the human leptin receptor were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin and 100 µg/ml streptomycin. Chinese hamster ovary (CHO) cells were cultured in Ham’s F-12, supplemented with 10% FCS and 100 units/ml penicillin and 100 µg/ml streptomycin. HEK293 cells and CHO cells were cultured at 37 ºC in a humidified atmosphere containing 5% CO₂ in air. For all leptin stimulations, recombinant leptin was used at a final concentration of 200 ng/ml.

Cloning, expression and purification of recombinant PTPs

cDNA cloning of the catalytic domains of wild-type (wt) PTP1B, SHP-2, PTPα domain 1 and CD45 domain 1–2, were performed as described previously (Andersen et al. 2000, Iversen et al. 2001). The PTP LAR expression vector was a kind gift from M Streuli, Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA, USA. All constructs were inserted into pGEX-5X expression vectors (Amersham Pharmacia Biotech). All coding sequences were confirmed by DNA sequencing. Expression and purification of the glutathione S-transferase (GST) fusion proteins were performed as described previously (Iversen et al. 2001, Lund et al. 2004).

Transient transfection assays, β-galactosidase and chloramphenicol acetyl transferase (CAT) assays

CHO cells were seeded (4 × 10⁵) in 60 mm tissue culture dishes (Nunc, Roskilde, Denmark) in Ham’s F-12 medium supplemented with 10% FCS, 100 units/ml penicillin and 100 µg/ml streptomycin at 37 ºC. Twelve-hour prior to transfection, cells were washed twice and incubated in 3 ml serum-free GC3 medium (1:1 mixture of Dulbecco’s modified Eagles’ medium and Ham’s F-12, supplemented with 10 µg/ml transferrin, 160 mU/ml insulin, 2 mM t-glutamine, 2 mM non-essential amino acids, 100 units/ml penicillin and 100 µg/ml streptomycin). Cells were transiently transfected by the calcium phosphate precipitation method as described previously (Chen & Okayama 1987) with the following amounts for each expression vector (per dish) in Opti-MEM medium: 3 µg SV40/β-galactosidase (pCH110 plasmid from Pharmacia Biotech Inc.), 1·5 µg Insulin promoter gamma-like element (Ins-GLE) CAT reporter construct (Galsgaard et al. 1996), 1·5 µg leptin receptor and 0·25–2·0 µg PTP

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(expression plasmids containing cDNAs encoding the various PTP forms have been inserted into the pcDNA3-1+ vector from Invitrogen). As control, the empty pcDNA3-1+ vector was used. Cells were incubated for 4 h, washed and subsequently cultured in serum-free GC3 medium in the absence or presence of 200 ng/ml leptin. After 24 h, cells were harvested and the β-galactosidase activity of each cellular extract was measured as follows: 10 μl cellular extract was mixed with 1 ml β-gal buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl and 1 mM MgSO₄) supplemented with β-mercaptoethanol and O-nitrophenyl-β-n-galacto-pyranoside. The reaction mixture was incubated at 37 °C for 2 h and absorbance was measured at 420 nm, and used for normalisation of transfection efficiency (i.e. internal control) before determination of CAT activity. The CAT assay was performed as previously described (Galsgaard et al. 1996). The concentration of leptin used in this assay (200 ng/ml) was found in dose–response experiments to give maximal stimulation of transcription. All experiments were performed in duplicate and repeated at least three times.

**Generation of phosphorylated GST-JAK2 fusion proteins**

The cDNA encoding the tyrosine kinase domain of JAK2 was amplified by PCR from a plasmid containing the cDNA encoding the full-length JAK2. The following primers were used: 5’-oligonucleotide sense primer with a BamHI restriction enzyme site at its 5’-end; 5’-atagg gatecCCAAAACCACAGGCACCTT-3’ and an antisense 3’-oligonucleotide primer with an XhoI restriction enzyme site at its 5’-end; 5’-ccgctcgagCTGCATGA TTGTGTT-3’. The PCR product was digested with BamHI and XhoI and the BamHI/XhoI fragment encoding the JAK2 amino acid residues (850–1132) was ligated into the GST fusion vector pGEX-5X-3 (Pharmacia Biotech Inc.). The coding sequence of the resulting plasmid was confirmed by DNA sequencing. Induction and affinity purification of the GST-JAK2 fusion proteins was performed as recommended by the manufacturer (Pharmacia Biotech Inc.) using the Epicurian coli TKX1 strain that harbours a plasmid encoding an inducible tyrosine kinase (elk) gene as recommended by the manufacturer (Strategene, La Jolla, CA, USA).

**Dephosphorylation of pTyr-GST-JAK2 and pTyr-STAT3 proteins in vitro**

Preparations of pTyr-GST-JAK2 fusion proteins (400 ng protein) or nuclear extracts from HEK293 cells containing pTyr-STAT3 proteins (5 μg protein) were incubated with different recombinant PTPs in assay buffer (20 mM Hepes, 50 mM NaCl and 1 mM EDTA, pH 6.5; final volume of 16 μl) for the indicated time-periods (0:5–20 min) at 25 °C. The reaction was stopped by the addition of 4 μl 5 × SDS sample buffer, followed by heating (10 min at 90 °C). The amount of recombinant PTP used in the assay was normalised according to the catalytic PTP activity, determined by hydrolysis of p-nitrophenyl phosphate (pNPP; Sigma) (see below).

**Western blot analysis**

For detection of PTP-induced dephosphorylation of pTyr-JAK2 and pTyr-STAT3, the proteins were separated by use of the Novex electrophoresis system (Invitrogen), blotted onto PVDF membranes (Invitrogen) and visualised by the ECL plus detection system (Amersham Pharmacia Biotech) as previously described (Richter et al. 2003). The primary antibodies used were anti-JAK2 pTyr1007/pTyr1008 antibody (Biosource International, Camarillo, CA, USA) or anti-PTyr (4 G10; Upstate Biotechnology Inc., Lake Placid, NY, USA), and anti-pTyr 705 STAT-3 antibody (Cell Signaling Technology Inc., Beverly, MA, USA), both diluted in 1:1000 in TBST (50 mM Tris/HCl, pH 7.4, 150 mM NaCl and 0.1% Tween 20) with 0.25% bovine serum albumin. Goat anti-rabbit horseradish peroxidase-conjugated antibody (Amersham Pharmacia Biotech) diluted 1:3000 was used as secondary antibody. For the control of equal amounts of protein, the pTyr blots were stripped and re-probed with specific antibodies, i.e. anti-STAT3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; diluted 1:500) and anti-JAK2 (Upstate Biotechnology; diluted 1:5000), as previously described (Stofega et al. 2000).

**Measurement of PTP1B catalysed hydrolysis of pNPP**

The phosphatase activity was assayed using pNPP as substrate and carried out essentially as described previously (Iversen et al. 2001, Lund et al. 2004). The assay buffer (pH 7.4), consisting of 50 mM 3,3-dimethylglutarate, 1 mM EDTA, 5 mM glutathione and 0.5% FCS (not heat-inactivated) was adjusted to an ionic strength of 0.15 M by the addition of NaCl. In brief, appropriately diluted inhibitors (undiluted and 50 μM compound) were added to the reaction mixture containing 0 or 2.5 mM pNPP (final assay concentration, total volume of 100 μl). The reaction was initiated by the addition of the enzyme (i.e. recombinant PTP1B) and allowed to proceed for 5 min before the inhibitor was added and the time was recorded, i.e. continued incubation for 5–60 min at 37 °C. The reaction was stopped by the addition of 20 μl 0.5 M NaOH in 50% ethanol. The enzyme activity was determined by measuring the absorbance at 405 nm using a Spectra MAX384 microplate spectrophotometer (Molecular
Devices Inc., Sunnyvale, CA, USA) with appropriate corrections for absorbance of substrate, compounds and non-enzymatic hydrolysis of substrate. The reported standard deviation values were calculated from at least four independent experiments.

**Statistical analyses**

Data are presented as means ± S.D. Statistical analyses were performed using GraphPad Prism software. Statistical significance was tested with two-tailed unpaired t-test. Differences were considered significant if \( P < 0.05 \).

**Nuclear extracts and electrophoretic mobility shift assay (EMSA)**

The HEK293 cells were seeded in 10 cm tissue culture dishes (Nunc) in RPMI 1640 medium supplemented with 10% FCS, 100 units/ml penicillin and 100 µg/ml streptomycin at 37 °C. After an overnight starvation in RPMI 1640 supplemented with 0.5% FCS, 100 units/ml penicillin and 100 µg/ml streptomycin, the cells were preincubated with 50 µM 2-(ethoxyoxalylamino)-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-3-carboxylic acid ethyl ester (trifluoroacetate) (compound 2) for 30 min, followed by the addition of 200 ng/ml leptin. After incubation for 0, 10 or 30 min at 37 °C, the culture dishes were placed on ice, and the cells were washed twice with ice-cold PBS and lysed in buffer A (20 mM Hepes, pH 7.9, 10 mM KCl, 1 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM 4-(2-aminoethyl)benzene sulfonyl fluoride, 1 mM sodium orthovanadate, 1 µg/ml aprotinin, 1 µg/ml leupeptin and 20% glycerol) containing 0.5% triton X-100. After 5 min of incubation on ice, the nuclei were collected by centrifugation at 2500 g for 7 min at 4 °C, resuspended in 5 volumes of a hypertonic buffer (buffer A containing 400 mM NaCl), and incubated on a rocking platform for 30 min at 4 °C. The supernatant was collected after centrifugation at 20 000 g for 30 min at 4 °C. The double-stranded m67 serum inducible element (SIE) oligonucleotide (5'-aaggTTATTTCCCGTAAATCCCTA-3') was 32P-labelled in a fill-in reaction using [γ-32P]-dCTP and DNA polymerase (Klenow fragment). An approximately 20 fmol probe was used per reaction with 10 µg nuclear extract in EMSA buffer (100 mM Hepes, pH 7.9, 10 mM NaCl, 1 mM MgCl₂, 1 mM EDTA and 10% (v/v) glycerol) containing 0.1 µg/µl double-stranded poly dI/dC (polydeoxyinosinic-deoxycytidylic acid; Amersham Pharmacia). The EMSA reactions were preincubated for 30 min at 30 °C prior to separation on a 5% polyacrylamide gel containing 2% glycerol and 0.25% TBE (25 mM Tris/ HCl, 25 mM boric acid and 0.25 mM EDTA, pH 7.9). The gel was dried and visualised by autoradiography and quantified by ImageQuant analysis (Molecular Dynamics, Sunnyvale, CA, USA). The specificity of the complex was demonstrated by competition with increasing amounts of either cold specific oligonucleotide m67 SIE (see above) or non-specific oligonucleotide (μCG; i.e. 5'-GATGAAAT TGACGTCATGGTAAAA-3') (Galsgaard et al. 1996) (not shown). In addition, the presence of STAT3 in this complex was confirmed upon preincubation of the nuclear extracts with anti-STAT3 antibody, which resulted in a super-shift band (not shown).

**Compound synthesis**

The preparation of 2-(oxalamino)-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-3-carboxylic acid (compound 1) and of its prodrug ester, compound 2, have been described previously (Iversen et al. 2000, Andersen et al. 2002).

**Results**

The precise molecular events involved in the regulation of transmission, duration and termination of leptin signal transduction remain to be fully elucidated. Compelling evidence suggests JAK2 and STAT3 as important signalling molecules in the mediation of leptin-induced effects in the central nervous system and the peripheral tissues (Banks 2003, Cui et al. 2004). Results from studies of PTP1B-deficient mice and cell lines derived therefrom suggest a key role of PTP1B in the control of leptin and insulin signalling (Elchebly et al. 1999, Klaman et al. 2000, Cheng et al. 2002, Zabolotny et al. 2002), which is further supported by studies pointing towards JAK2 as a bona fide substrate of this PTP (Myers et al. 2001). We therefore set out to investigate if PTP1B mediates the cessation of the leptin signal transduction by direct targeting of signalling molecules such as JAK2 and STAT3.

**Selective and dose-dependent inhibition of leptin-induced transcription by PTP1B**

The effect of PTP1B on leptin-induced STAT3-dependent transcription was analysed using transient transfection of CHO cells with the STAT3-responsive proximal part of the insulin promoter linked to a CAT reporter. A 6-fold stimulation of leptin-induced transcription was observed in control cells (Fig. 1). Co-transfection with either wt PTP1B or a truncated form of PTP1B (PTP1B1-321) (Tonks et al. 1988) resulted in a significant and dose-dependent suppression of leptin-induced transcription. PTP1B1-321 seemed slightly more potent in inhibiting reporter activity compared with wt PTP1B, in that only half the amount of PTP construct was necessary to obtain complete inhibition (1 µg versus 2 µg of PTP construct). A so-called substrate-trapping mutant of
PTP1B, PTP1B\textsubscript{D181A} (Flint et al. 1997), which displays very weak catalytic activity, was not able to inhibit the leptin-induced CAT activity at any dose tested (0.25–2 µg PTP construct) (Fig. 2), thus demonstrating that the PTP1B catalytic activity is required for inhibition. In addition to PTP1B, previous reports have provided evidence supporting a negative regulatory role of both LAR (Ahmad et al. 1997, Zabolotny et al. 2004) and PTP\textsubscript{α} (Møller et al. 1995, Cong et al. 1999, Calera et al. 2000) in insulin signalling, though the exact level at which these phosphatases affect the signal transmission remains to be fully elucidated. As with PTP1B, the phosphatases SHP-2 (Li & Friedman 1999, You et al. 1999, Yu et al. 2000) and CD45 (Irie-Sasaki et al. 2001, Yamada et al. 2002) seem to influence different signalling events by regulation of the JAK/STAT pathways. To evaluate whether the observed reduction in reporter activity was specific to PTP1B or a common feature shared by distantly related PTPs, we co-transfected the CHO cells with LAR, PTP\textsubscript{α}, SHP-2 or CD45 at doses up to 2 µg PTP construct and measured the STAT3-dependent reporter activity. With the exception of LAR, neither of the PTPs was able to inhibit leptin-induced CAT activity (Fig. 3). However, co-transfection with 2 µg LAR construct resulted in an approximately 50% reduction of leptin-stimulated reporter activity.

**JAK2 and STAT3 are dephosphorylated by PTP1B in vitro**

To identify the mechanism by which PTP1B mediates its inhibitory effect on leptin signalling, we tested the ability of recombinant PTP1B and other PTPs to dephosphorylate JAK2 and STAT3 in vitro, i.e. in preparations of phosphorylated GST-JAK2 fusion proteins and in nuclear extract isolated from leptin-stimulated HEK293 cells respectively. A significant and time-dependent dephosphorylation of both JAK2 and STAT3 proteins was induced by PTP1B, resulting in an approximately 90% reduction in the phosphorylation level after 20 min (Fig. 4). In the presence of LAR, the STAT3 proteins but not the JAK2 proteins were slightly dephosphorylated (Fig. 4B). In contrast, CD45 and PTP\textsubscript{α} were inefficient in catalysing dephosphorylation of both JAK2 and STAT3 proteins, although we have previously demonstrated that these recombinant PTP constructs have full activity against pTyr substrates under similar conditions (Iversen et al. 2000, Lund et al. 2004).

**Inhibition of PTP1B enhances STAT3 activity**

To further access the specific role of PTP1B, we decided to analyse leptin-induced STAT3 DNA binding in cells using a selective PTP1B inhibitor, compound 1.
This compound inhibits PTP1B and its close homologue T-cell (TC)-PTP at pH 7.0 with inhibitor constant ($K_i$) values of 4.7 and 9.4 µM respectively (Andersen et al. 2002). As compound 1 was previously shown to display limited cell permeability, we decided to use a prodrug of compound 1, i.e. compound 2 (Fig. 5A), for which we have demonstrated an efficient uptake by Madin–Darby canine kidney cell monolayers – a standard cell line used to measure passive uptake in cells (Andersen et al. 2002). However, we also demonstrated a rapid hydrolysis of compound 2 upon exposure to serum. To test if compound 2 was sufficiently stable to allow uptake in our target cells as a prodrug, we investigated the kinetic properties of this inhibitor under conditions similar to those present in cellular systems. While compound 1 inhibited PTP1B

![Figure 2](image_url)

**Figure 2** The catalytic activity of PTP1B is required for inhibition of leptin signalling. CHO cells (4x10⁵) were transiently transfected with expression plasmids encoding the leptin receptor (1.5 µg), a STAT3-responsive CAT reporter (1.5 µg), β-galactosidase (3 µg) and the indicated amount of wt PTP1B, the truncated form PTP1B₁₋₃₂₁ or the catalytically inactive mutant PTP₁B₁₈₁₆₈ as indicated on the graph. Cells were stimulated with 200 ng/ml leptin for 24 h and CAT and β-galactosidase activities were measured. The fold induction by leptin of CAT activity normalised to β-galactosidase activity is shown. The means with s.d. of three independent experiments are shown.

![Figure 3](image_url)

**Figure 3** Selective PTP1B inhibition of leptin-induced transcription. CHO cells (4x10⁵) were transiently transfected with expression plasmids encoding the leptin receptor (1.5 µg), a STAT3-responsive CAT reporter (1.5 µg), β-galactosidase (3 µg) and PTP (i.e. PTP1B, SHP-2, CD45, PTPα or LAR as indicated on the graph). Cells were stimulated with 200 ng/ml leptin for 24 h and CAT and β-galactosidase activities were measured. The fold induction by leptin of CAT activity normalized to β-galactosidase activity is shown. The means with s.d. of three independent experiments are shown.
Figure 4 STAT3 and JAK2 are dephosphorylated by PTP1B in vitro. (A) Preparations of pTyr-JAK2-GST fusion proteins (400 ng) were incubated with recombinant PTPs for the indicated time-intervals. The JAK2 tyrosine phosphorylation status was determined by Western blotting using anti-pTyr antibody. The figure shows the time-dependent dephosphorylation of JAK2 following incubation with the indicated phosphatase. (B) Nuclear extracts (5 µg) from leptin-stimulated HEK293 cells were incubated with recombinant PTPs for the indicated time-intervals. The STAT3 tyrosine phosphorylation status was determined by Western blotting using anti-pTyr 703 STAT3 antibodies. The figure shows the time-dependent dephosphorylation of STAT3 following incubation with the indicated phosphatase. Equal amounts of phosphatase activity based on pNPP hydrolysis were used in both assays. Representative blots are shown.
throughout the incubation time, the inhibition by compound 2 was markedly time-dependent, with increased inhibition of the PTP1B-catalysed hydrolysis of pNPP as a function of time (Fig. 5A). This result demonstrated the presence of a slow hydrolysis of compound 2, affording the active di-acid inhibitor (compound 1, schematic illustration in Fig. 5A). In conclusion, these studies demonstrated that the prodrug compound 2 is sufficiently stable for passage of the cell membrane under the given assay conditions, as well as being sufficiently labile for intracellular release of the active PTP1B inhibitor, compound 1, and thus appropriate for application to our target cells.

HEK293 cells stably transfected with the leptin receptor constitute a suitable cellular model system, in which STAT3-dependent DNA binding activity was induced by leptin after 10 and 30 min of stimulation, as measured by EMSA of nuclear extracts (Fig. 5B). Incubation of the cells in the presence of compound 2 clearly enhanced the response to leptin, by approximately 2·5-fold (Fig. 5C), indicating an inhibition of PTP1B and thus a role of this endogenous phosphatase in the suppression of leptin-induced STAT3 activity. It should, however, be noted that the enhanced leptin-induced STAT3 activity observed in the presence of compound 2 cannot be differentiated to an effect on either PTP1B or TC-PTP, as the parent compound displays similar affinity towards the two PTPs. Collectively, these results provide evidence for the negative regulatory role of PTP1B on leptin signalling to be mediated by dephosphorylation of JAK2 and STAT3, subsequently leading to inactivation at the level of both these important proteins.

Discussion
Genetic and biochemical evidence for the important role of PTP1B as a negative regulator of insulin- and
leptin-induced metabolic actions have emerged in recent years. In particular, the increased insulin sensitivity, resistance to diet-induced obesity and augmented energy expenditure observed in PTP1B-deficient mice (Elchebly et al. 1999, Klaman et al. 2000), together with the enhanced phosphorylation levels of insulin and/or leptin signalling molecules \textit{in vivo} and in several cell lines (Elchebly et al. 1999, Klaman et al. 2000, Cheng et al. 2002, Zabolotny et al. 2002) have established an important link between the two signalling pathways and suggest PTP1B as a key therapeutic target in obesity and type 2 diabetes. In this study, we have provided evidence
for a negative regulatory role of PTP1B on leptin signalling, mediated through a direct and time-dependent dephosphorylation of both JAK2 and STAT3.

First, we identified PTP1B as a negative regulator of leptin signalling in CHO cells by demonstrating a significant and dose-dependent inhibition of leptin-induced transcription by wt PTP1B (Fig. 1). For these experiments, a leptin-responsive part of the insulin promoter was used and it should be noted that this promoter region is responsive to transcription factors other than STAT3, and thus that the observed effect of PTP1B might not be exclusively mediated through inhibition of the JAK/STAT pathway. Importantly, the inhibition required the catalytic activity but was independent of the presence of the C-terminal end of the enzyme, i.e. wt PTP1B and PTP1BΔ321 but not PTP1BΔ361A inhibited leptin-induced reporter activity (Fig. 2). A number of recent publications have illustrated reduced leptin signalling and decreased amounts of pTyr-STAT3 in the presence of PTP1B in various cellular systems, such as fibroblast and Cos-7 cells (Zabolotny et al. 2002), as well as the murine hypothalamic cell line, GT1–7 (Kasubska et al. 2002), while increased pTyr-STAT3 levels were observed in hypothalamic cell line, GT1–7 (Kasubska et al. 2002). In the present study, the incubation of nuclear extract containing pTyr-STAT3 proteins with recombinant PTP1B was demonstrated to result in a time-dependent dephosphorylation of STAT3 (Fig. 4C).

The PTP1B-mediated dephosphorylation of JAK2 is believed to occur at or near the endoplasmic reticulum, as Tremblay, Boisclair and colleagues have recently provided evidence for a direct interaction between the endoplasmic reticulum-bound form of PTP1B (Frangioni et al. 1992) and JAK2 proteins to be feasible (Gu et al. 2003). The sequence [D/E]-Y-Y-[R/K], demonstrated to constitute a consensus substrate recognition motif for PTP1B, is present in several proteins including JAK2, TYK2 (Myers et al. 2001) and the insulin receptor (Salmeen et al. 2000). Interestingly, a similar motif with either mono or tandem tyrosine residues preceded by acidic residues, indicative of a potential genuine substrate for PTP1B action, is absent in STAT3 proteins. However, other proteins have been demonstrated to serve as substrate for PTP1B using a different motif, such as the SH3 domain in p130cas protein, assumed to interact with proline-rich stretches of PTP1B (Yu et al. 1994, Liu et al. 1996). While the cellular compartment within which PTP1B induces dephosphorylation of STAT3 remains to be determined, a recent study has indicated that prolactin-induced STAT5 phosphorylation and nuclear translocation is reduced upon over-expression of PTP1B in COS7 cells (Aoki & Matsuda 2000), suggesting that the PTP1B-induced dephosphorylation of STAT3 observed in the present study occurs in the cytosol.

Valuable information on the function of specific genes at the molecular level can be obtained from cell lines, such as (i) cancer cell lines with specific chromosomal deletions and (ii) cell lines derived from knockout animals. Similarly, gene silencing with RNA interference and oligonucleotide antisense techniques have provided important information (Blaskovich & Kim 2002, Andersen et al. 2004, Dubé & Tremblay 2004). However, these approaches have certain limitations as counter-regulatory mechanisms such as up- or down-regulation of other genes may mask the functionality of the gene in question and thus prevent a correct interpretation of the situation. Similar compensatory mechanisms may be at play in antisense studies, which in addition depend on the turnover of the gene product, i.e. a long half-life of a specific protein may prevent the use of this technique in primary cells. To analyse in more detail the effect of PTP1B as a negative regulator of leptin signalling, we have used a highly selective inhibitor of PTP1B. In contrast to the above approaches, such inhibitors allow direct assessment of the function of PTP1B – provided that sufficient specificity for the particular enzyme can be obtained.

We have previously demonstrated that compound 1 is highly selective for PTP1B and the homologous TC-PTP (Iversen et al. 2002). Using a prodrug of compound 1, we have here demonstrated a significant increase in leptin-induced STAT3 DNA binding activity, most likely produced by specific inhibition of endogenously expressed PTP1B and/or TC-PTP.

While recent studies have identified TC-PTP as a phosphatase responsible for dephosphorylation of STAT1 in the nucleus (ten Hoeve et al. 2002), it was found that PTP1B, but not TC-PTP, could attenuate growth hormone-mediated JAK2 dephosphorylation (Gu et al. 2003). Thus, despite the fact that PTP1B and TC-PTP have a sequence identity of 74% in the catalytic domains (Iversen et al. 2002), it seems that subtle structural differences and/or distinct subcellular localisation are responsible for the reported different substrate specificities and dissimilar biological functions of these highly homologues PTPs (Tiganis et al. 1998, Simoncic et al. 2002, Gu et al. 2003, Pasquali et al. 2003, Persson et al. 2004). This difference is also evident from the strikingly different phenotypes of PTP1B- versus TC-PTP-deficient mice (You-Ten et al. 1997, Elchebly et al. 1999, Kliman et al. 2000, Heinonen et al. 2004).

operate on distinct cytokine-induced signal transduction cascades. In the present study, PTPα, SHP-2 and CD45 did not influence leptin-stimulated transcription or the phosphorylation level of JAK2 and STAT3, indicating a selective action of PTP1B on leptin signalling. The lack of activity of CD45 on JAK2 and STAT3 is in contrast to a previous study, in which CD45 was shown to be a potential negative regulator of JAK proteins in haematopoietic cell types (Irie-Sasaki et al. 2001). This discrepancy may be attributable to the fact that different cellular systems and stimulatory factors were used, as compared with the current conditions.

The present study has demonstrated a reduced leptin-induced transcription and a time-dependent dephosphorylation of STAT3 in the presence of LAR, suggesting a potential role of this PTP in the regulation of leptin signalling. An inhibitory effect of LAR on pTyr-STAT3 or any other members of the JAK/STAT pathway has not been previously illustrated, although prior reports describing potential cross-talk between leptin and insulin signalling and JAK/STAT proteins may support such an effect of LAR on STAT3 (Ahmad & Goldstein 1995, Ahmad et al. 1997, Cheng et al. 2002, Zabolotny et al. 2002, 2004).

In summary, we have identified PTP1B as a negative regulator of the leptin signalling, most likely mediated by direct interaction with both JAK2 and STAT3. Importantly, these findings were supported by experiments with a selective PTP1B inhibitor prodrug, which significantly enhanced leptin-induced STAT3 activation compared with the current conditions.

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