Identification of YB-1 as a regulator of *PTP1B* expression: implications for regulation of insulin and cytokine signaling

Toshiyuki Fukada and Nicholas K. Tonks¹

Cold Spring Harbor Laboratory, 1 Bungtown Road,
Cold Spring Harbor, NY 11724, USA

¹Corresponding author
e-mail: tonks@cshl.edu

Changes in expression of PTP1B, the prototypic protein tyrosine phosphatase, have been associated with various human diseases; however, the mechanisms by which PTP1B expression is regulated have not been defined. We have identified an enhancer sequence within the *PTP1B* promoter which serves as a binding site for the transcription factor Y box-binding protein-1 (YB-1). Overexpression of YB-1 resulted in increased levels of PTP1B. Furthermore, depletion of YB-1 protein, by expression of a specific antisense construct, led to an ~70% decrease in expression of PTP1B, but no change in the level of its closest relative, TC-PTP. Expression of antisense YB-1 resulted in increased sensitivity to insulin and enhanced signaling through the cytokine receptor gp130, which was suppressed by re-expression of PTP1B. Finally, we observed a correlation between the expression of PTP1B and that of YB-1 in cancer cell lines and an animal model of type II diabetes. Our data reveal an important role for YB-1 as a regulator of PTP1B expression, and further highlight PTP1B as a critical regulator of insulin- and cytokine-mediated signal transduction.

Keywords: cytokine/insulin/phosphorylation/PTP1B/YB-1

Introduction

The reversible phosphorylation of tyrosyl residues in proteins is a critical element of the cellular responses to environmental stimuli, including growth factors, cytokines and hormones, which culminate in such functions as growth, proliferation, differentiation, metabolism and migration. Protein tyrosine phosphorylation is controlled through the coordinated actions of protein tyrosine kinases (PTKs) and phosphatases (PTPs). Not unexpectedly, disturbance of the normal balance between PTK and PTP function results in aberrant tyrosine phosphorylation and has been implicated in the etiology of several human diseases, including cancer, diabetes and inflammation (Tonks and Neel, 2001). Although initially considered in terms of a housekeeping function, recent attention has been drawn to the importance of PTPs as critical regulators of signal transduction. The PTPs are represented by a structurally diverse family of ~100 members that encompasses both receptor-linked and non-transmembrane enzymes (Andersen *et al.*, 2001). Various strategies,

from the development of substrate-trapping mutants to gene ablation, have highlighted the potential for PTPs to display exquisite substrate specificity *in vivo* and to function as regulators of fundamentally important signal transduction events (Tonks and Neel, 2001).

The prototypic member of the PTP family is the enzyme PTP1B. This enzyme, named from a pool of PTP activity resolved by ion-exchange chromatography, originally was purified from human placenta as a 37 kDa catalytic domain (Tonks *et al.*, 1988). However, the full-length form of the protein also contains a regulatory C-terminal segment (Brown-Shimer *et al.*, 1990; Chernoff *et al.*, 1990; Guan and Dixon, 1990) that functions in targeting the enzyme to the cytoplasmic face of membranes of the endoplasmic reticulum (Frangioni *et al.*, 1992). PTP1B has been implicated in the regulation of a number of signaling pathways, in particular those involving tyrosine phosphorylation induced by growth factors, cytokines and hormones (Tonks and Neel, 2001). For example, the use of substrate-trapping mutant forms of PTP1B identified the epidermal growth factor (EGF) receptor as a physiological target (Flint *et al.*, 1997). Interestingly, it has been shown, through the use of fluorescence resonance energy transfer, that PTP1B acts specifically on receptor-PTKs that have undergone endocytosis, indicating that PTP1B acts to downregulate the growth factor signal rather than controlling the basal phosphorylation status of the receptor (Haj *et al.*, 2002). Mice in which the gene for PTP1B has been ablated display enhanced sensitivity to insulin and a resistance to obesity induced by a high fat diet (Elchebly *et al.*, 1999). Interestingly, both basal metabolic rate and total energy expenditure are enhanced in PTP1B-deficient mice (Klamann *et al.*, 2000). These effects coincide with enhanced phosphorylation of the insulin receptor. The structural basis for the recognition of the insulin receptor as a substrate by PTP1B has now been determined (Salmeen *et al.*, 2000), highlighting the importance of the motif E/D-pY-pY-K/R for high affinity interaction. This motif was also detected in the JAK family of PTKs, from which Tyk2 and Jak2 were shown to be substrates of PTP1B (Myers *et al.*, 2001). Interestingly, the dephosphorylation of JAK2 is the mechanism by which PTP1B influences signaling through the leptin receptor and thus may be implicated in leptin resistance associated with obesity (Cheng *et al.*, 2002; Zabolotny *et al.*, 2002). As a result of these various studies, a picture is now emerging of PTP1B as a critical regulator of signaling in normal and disease states. In light of this importance, one would anticipate that the activity of PTP1B would be tightly controlled *in vivo*. An understanding of such control mechanisms will be essential to achieve an understanding of the regulation of several major signal transduction pathways.

The activity of PTP1B is regulated by post-translational modification. Phosphorylation of PTP1B on either serine (Flint *et al.*, 1993) or tyrosine (Dadke *et al.*, 2001; Tao *et al.*, 2001b) residues has been implicated in up- or downregulation of activity. More recently, attention has

been drawn to reversible oxidation of the active site cysteine residue as a regulatory mechanism (Lee *et al.*, 1998; Mahadev *et al.*, 2001; Meng *et al.*, 2002). Limited proteolysis of PTP1B, which would be anticipated to alter both activity and subcellular distribution of the

Fig. 1. Identification of a transcriptional enhancer element in the human *PTP1B* promoter. (A) Schematic representation of the human *PTP1B* promoter. The human *PTP1B* gene lacks a TATA box, an initiator or a downstream promoter element. A p210 Bcr-Abl-responsive sequence (PRS) is located at -49 to -37 bp from the transcription start site in the human *PTP1B* promoter and serves as a binding site for the transcription factors Egr-1, Sp1 and Sp3. A second element with potential enhancer function was identified at -167 to -151 bp from the transcription start site. (B) Schematic representation of the reporter constructs used to define the limits of the enhancer sequence in the *PTP1B* promoter. Constructs were generated, as described in Materials and methods, in which the indicated sequences from the human *PTP1B* promoter drove expression of a luciferase reporter by enhancing SV40 promoter activity. (C) Identification of the sequence between -155 and -132 bp from the transcription start site of the *PTP1B* promoter as the minimum sequence required for enhancer function. Rat1 cells were transiently transfected with 1 μ g of either the empty vector (pGL3-promoter), the control plasmid, in which SV40 promoter activity is driven by the SV40 enhancer sequence (pGL3-control), or the luciferase reporter constructs, in which SV40 promoter activity is driven by sequences from the human *PTP1B* promoter. A 1 μ g aliquot of pRL-TK was also included to normalize transfection efficiency. Cells were harvested, lysed and assayed for luciferase activity. Values were normalized for transfection efficiency and represent the mean $\pm$ SD of three separate experiments.

phosphatase, has been described (Frangioni *et al.*, 1993). In addition, control is exercised at the level of expression. Changes in the level of expression of PTP1B have been noted in several human diseases, particularly those associated with disruption of the normal patterns of tyrosine phosphorylation (Wiener *et al.*, 1994a,b; Ahmad *et al.*, 1997; Dadke *et al.*, 2000). We have shown previously that the expression of PTP1B is induced specifically by the p210 Bcr-Abl oncoprotein, a PTK that is directly responsible for the initial manifestations of chronic myelogenous leukemia (CML) (LaMontagne *et al.*, 1998a,b). We identified a p210 Bcr-Abl-responsive sequence (PRS) in the human *PTP1B* promoter, which is important for stimulation of activity in response to the oncoprotein PTK (Fukada and Tonks, 2001). The PRS is recognized by Egr-1 and Sp C₂H₂ zinc finger transcription factors, which act in a reciprocal manner to regulate the expression of PTP1B in response to the p210 Bcr-Abl oncoprotein (Fukada and Tonks, 2001). In the same study, we identified another motif, which displays features of a recognition site for GATA-binding proteins. Disruption of this motif inhibited promoter activity by ~60% in either the presence or absence of p210 Bcr-Abl, suggesting that this sequence may act as a general enhancer element.

In order to gain a more complete understanding of the transcriptional control of *PTP1B* gene expression, we have defined and characterized this enhancer element in the current study. Using a combination of electrophoretic mobility shift assays (EMSAs) and protein purification, we purified a *PTP1B* enhancer-binding protein and identified it, by primary sequence determination, as YB-1, which belongs to the Y box-binding protein family (Wolffe, 1994). We observed that depletion of cellular YB-1, by expression of a specific antisense YB-1 construct, resulted in decreased expression of PTP1B, whereas overexpression of YB-1 led to an increase in the level of PTP1B. Interestingly, the antisense YB-1-induced decrease in expression of PTP1B resulted in enhancement of insulin and cytokine signaling responses, which was counteracted by re-expression of PTP1B. Finally, we have observed a correlation between the expression of PTP1B and that of YB-1 in cancer cell lines and an animal model of type II diabetes. Our data illustrate the importance of YB-1 in the regulation of PTP1B expression and suggest a novel aspect to control of insulin- and cytokine-mediated signaling via the level of expression of PTP1B *in vivo*.

Results

Identification of an enhancer sequence in the *PTP1B* promoter

Previously, we initiated a characterization of the *PTP1B* promoter and identified two regulatory elements. We demonstrated that the p210 Bcr-Abl oncoprotein tyrosine kinase induces increased expression of PTP1B, exerting effects at the transcriptional level through a PRS (−49 to −37 bp upstream from the transcription start site) (Figure 1A). The PRS recognizes Egr-1 and Sp family C₂H₂ zinc finger transcription factors, which play a reciprocal role in the control of expression from the *PTP1B* promoter in response to p210 Bcr-Abl (Fukada and Tonks, 2001). We also noted a sequence element between −167 and −151 bp upstream from the transcription start site

which displays features of a recognition site for GATA family-binding proteins and which enhanced promoter activity in the presence or absence of p210 Bcr-Abl. We have characterized this latter element in the current study.

In order to define the enhancer sequence, we created a series of reporter plasmids in which deletion mutants from the *PTP1B* promoter were linked to the minimal SV40 promoter and drove expression of a luciferase reporter gene by enhancing SV40 promoter activity (Figure 1B). Luciferase activity was measured following transient expression of the reporter plasmids in Rat1 cells and compared with the activity of SV40 minimal promoter–luciferase driven by the SV40 enhancer (pGL3-control, Figure 1C, lane 2). We observed that the *PTP1B* promoter activated expression of the SV40 minimal promoter–luciferase construct (pGL3-promoter) to a similar extent to the SV40 enhancer (Figure 1C, lanes 2 and 3). Deletion of the sequence upstream from −168 bp did not affect *PTP1B* enhancer activity (Figure 1C, lane 4). However, activity was abolished by further deletion of the sequence from −167 to −152 bp (Figure 1C, lane 5), suggesting that components of this sequence are essential for enhancer function. Deletion of the sequence 3' from −132 bp did not inhibit enhancer activity, indicating that the segment from −167 to −132 bp was sufficient for enhancer function (Figure 1C, lane 6). In order to define the limits of the enhancer more precisely, we introduced shorter deletions into this active fragment. Using this approach, we identified a minimal element located between −155 and −132 bp upstream from the transcription start site that was sufficient for enhancer activity (Figure 1C, lane 7). Further deletions, either internally or at the termini of this minimal element, abrogated enhancer function (Figure 1C, lanes 8–12). Thus the sequence between −155 and −132 bp is necessary and sufficient to enhance promoter activity and may play a role in regulation of *PTP1B* gene expression.

Identification of proteins that interact with the *PTP1B* enhancer

We used several double-stranded DNA sequences derived from the *PTP1B* promoter as probes in EMSAs to visualize the interaction between the *PTP1B* enhancer and potential binding proteins. When incubated with nuclear extracts of Rat-1 cells, we observed the formation of a protein–DNA complex, termed complex A, with probes equivalent to sequences that displayed enhancer activity, i.e. −167 to −132 bp (−167/−132) and −155 to −132 bp (−155/−132) (Figure 2A). In contrast, dramatically lower levels of complex A were recovered when probes were used that were equivalent to inactive mutant forms of the *PTP1B* enhancer, i.e. −167 to −144 bp (−167/−144) and −167 to −132 bp containing an internal deletion from −155 to −144 bp [−167/−132 (Δ−155/−144)] (Figure 2A). Similarly, a second minor complex (complex B) of much lower abundance was also observed only with probes equivalent to active forms of the *PTP1B* enhancer. These data suggest that complex A may contain proteins that regulate the function of the enhancer element within the *PTP1B* promoter.

In order to identify the proteins that form the complexes with the *PTP1B* enhancer sequence, we implemented a purification strategy in which nuclear extract from Rat1

cells was subjected to ion exchange chromatography on SP-Sepharose, followed by affinity chromatography on an immobilized *PTP1B* enhancer probe. Purified proteins were visualized by SDS-PAGE, with recovery and specific activity determined by EMSA, using 32 P-labeled, -167/-132 probe (Figure 2B). Following this procedure, and using ~50 mg of nuclear extracts of Rat1 cells, we recovered ~41% of complex A and enriched the specific activity 62 000-fold after affinity chromatography on a

PTP1B enhancer-derived DNA sequence. The fractions eluted from the DNA affinity chromatography column with 0.8 M NaCl were concentrated, and ~5 μ g of protein was recovered. A portion (2 μ g) was then resolved further by SDS-PAGE. Proteins in the fraction were visualized with Coomassie Brilliant Blue G-colloidal concentrate, revealing primarily a single protein of mol. wt ~47 kDa (Figure 2C). This protein band was excised from the gel and subjected to tryptic digestion, followed by sequence

Fig. 2. Purification of YB-1 as a *PTP1B* enhancer-binding protein. (A) Formation of DNA-protein complexes on probes derived from the *PTP1B* enhancer. An EMSA was performed with or without nuclear extracts of Rat1 cells using the probes as indicated in (A). Double-stranded oligonucleotides were end labeled with the 5' end labeling kit (Pharmacia). Each probe (1×10^5 c.p.m.) was incubated in binding buffer with 5 μ g of nuclear extract from Rat1 cells at 25°C for 20 min. The positions of free probe and DNA-protein complexes are indicated by arrows. (B) Elution of proteins bound to the *PTP1B* enhancer double-stranded DNA probe affinity column. The SP-Sepharose eluate was applied to the DNA affinity column (containing biotinylated DNA sequence between -167 and -132 bp from the transcription start site in the *PTP1B* promoter) in 0.2 M NaCl, and bound proteins were eluted stepwise with increasing salt concentrations. Binding was monitored by EMSA using a double-stranded probe of the DNA sequence between -167 and -132 bp. The arrows indicate the positions of protein-DNA complexes and free probe. (C) SDS-PAGE of purified *PTP1B* enhancer-binding protein. Rat1 cell nuclear extracts or concentrated 0.8 M NaCl eluate from the DNA affinity chromatography (2 μ g of each) were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue G-colloidal concentrate. The protein band migrating at 47 kDa was excised from the gel for sequence analysis. (D) Identification of the 47 kDa protein as Y box-binding protein-1. Sequence analysis was performed by tandem mass spectrometry at the Cold Spring Harbor Laboratory Protein Chemistry Shared Resource. Sequences of six peptides were obtained and are highlighted. These sequences were matched identically with those from rat Y box-binding protein, YB-1.

analysis by tandem mass spectrometry at the Cold Spring Harbor Laboratory Protein Chemistry Shared Resource. The data from this analysis revealed six peptides that were identical in sequence to rat Y box-binding protein, YB-1 (Figure 2D).

The DNA sequence CTGATTGG(C/T)(C/T)AA is known as a Y box and has been identified as a recognition motif for the family of Y box-binding proteins, which are expressed in a wide range of cell types and display a variety of functions in the context of gene regulation

Fig. 3. Binding of YB-1 to the *PTP1B* enhancer. **(A)** Binding of YB-1 to *PTP1B* enhancer sequences. YB-1, purified on the basis of its interaction with the *PTP1B* enhancer, was incubated with biotinylated double-stranded DNA probes and streptavidin–Sephacrose for 6 h at 4°C. The protein–DNA complexes were subjected to SDS–PAGE, followed by immunoblotting with anti-YB-1 antibody. The experiment was performed using four different probes (in which the residue numbers are given relative to the transcription start site in the promoter). The probes –167/–132 and –155/–132 represent sequences with enhancer activity and interact with YB-1, whereas the probes that lacked enhancer activity, –167/–144 and –167/–132 with an internal deletion Δ –155/–144, displayed greatly impaired binding of YB-1. **(B)** Levels of YB-1 and PTP1B in nuclear extracts from Rat1 and HepG2 cells. Upper panel: nuclear extracts were incubated with biotinylated double-stranded DNA probes and streptavidin–Sephacrose for 6 h at 4°C. The protein–DNA complexes were subjected to SDS–PAGE, followed by immunoblotting with anti-YB-1 antibody. The probe –155/–132 represents a sequence with enhancer activity. Lower panel: total cell lysates (20 μ g) of either Rat1 or HepG2 cells were immunoblotted with antibody to YB-1, PTP1B or actin. **(C)** Antibody to YB-1 supershifted the complex of the *PTP1B* enhancer probe (–155/–132) and purified YB-1 in an EMSA. Specific antibodies to YB-1, GATA1 or GATA2 were used to test for a supershift of the protein–DNA complex in an EMSA. Purified YB-1 was incubated with or without each antibody for 1 h at 4°C then the DNA probe was incubated with the antibody–YB-1 mixture at 25°C for 20 min. The positions of the antibody-supershifted complex and the complex between YB-1 and the *PTP1B* enhancer probe are indicated by arrows. **(D)** GST–YB-1 bound directly to the *PTP1B* enhancer sequence. Left panel: purified GST and GST–YB-1 (1 μ g of each) was subjected to SDS–PAGE followed by staining with Coomassie Brilliant Blue R250. Right panel: purified GST or GST–YB-1 was incubated with the DNA probe in the presence or absence of unlabeled competitor probe at 25°C for 20 min and DNA–protein complexes were visualized in an EMSA. The arrows indicate the positions of the complex between YB-1 and the *PTP1B* enhancer probe and of the free probe.

(Wolffe *et al.*, 1992; Wolffe, 1994). However, the *PTP1B* promoter does not contain an obvious Y box motif. Nevertheless, it is now apparent that Y box-binding proteins will recognize motifs with little similarity to the classical Y box (Wolffe *et al.*, 1992; Wolffe, 1994). Therefore, in order to confirm an interaction between YB-1 and the *PTP1B* enhancer, we used an antibody to YB-1. Immunoblotting of both nuclear extracts and the fraction purified by DNA affinity chromatography revealed an immunoreactive protein of ~47 kDa that co-migrated with the purified *PTP1B* enhancer-binding protein (data not shown). In addition, we performed DNA pull-down assays, incubating the fraction purified by

DNA affinity chromatography with double-stranded DNA probes equivalent to active or inactive forms of the *PTP1B* enhancer. The interaction of YB-1 was determined by immunoblotting of the DNA precipitate. We observed that probes equivalent to sequences that displayed enhancer activity (i.e. -167/-132 and -155/-132) bound YB-1, whereas the binding to probes that were equivalent to inactive mutant forms of the *PTP1B* enhancer [i.e. -167/-144 and -167/-132 (Δ -155/-144)] was substantially reduced (Figure 3A). In addition, we observed the interaction between YB-1 and the *PTP1B* enhancer not only in nuclear extracts prepared from Rat1 fibroblasts but also in those from HepG2 human hepatocellular

Fig. 4. Inhibition of PTP1B expression and *PTP1B* enhancer function by depletion of cellular YB-1. (A) Immunoblot analysis to assess expression levels of YB-1 and various PTPs in stable Rat1 cell lines expressing either YB-1 antisense constructs (clone numbers 12, 20 and 23) or control plasmid (Rat1-control). Total cell lysate (20 μ g) was applied to each lane and subjected to SDS-PAGE followed by immunoblotting with the indicated antibodies to YB-1, PTP1B, SHP-2, LAR and TCPTP. Immunoblots with anti-actin antibodies were included as a loading control. (B) The effect of depletion of cellular YB-1 on *PTP1B* promoter activity. Control Rat1 cells (black bars) and Rat1 cell clone 20, expressing the antisense YB-1 construct (gray bars), were transiently transfected with 1 μ g of either the pGL3-basic plasmid, containing the luciferase reporter, the pGL3-control plasmid, in which expression of the luciferase reporter is driven by the SV40 minimal promoter and enhancer elements, or the plasmids in which expression of the luciferase reporter was driven by sequences from the human *PTP1B* promoter. A 1 μ g aliquot of pRL-TK was also included to normalize transfection efficiency. Cells were harvested, lysed and assayed for luciferase activity. Values were normalized for transfection efficiency and represent the mean $\pm$ SD of three separate experiments. (C) The effect of depletion of cellular YB-1 on *PTP1B* enhancer activity. Control Rat1 cells (black bars) and Rat1 cell clone 20, expressing the antisense YB-1 construct (gray bars), were transiently transfected with 1 μ g of either the pGL3-promoter plasmid, in which expression of the luciferase reporter is driven by the SV40 minimal promoter, the pGL3-control plasmid, in which expression of the luciferase reporter is driven by the SV40 minimal promoter and enhancer elements, or plasmids in which *PTP1B* enhancer sequences drove expression of luciferase from the SV40 minimal promoter. A 1 μ g aliquot of pRL-TK was also included to normalize transfection efficiency. Cells were harvested, lysed and assayed for luciferase activity. Values were normalized for transfection efficiency and represent the mean $\pm$ SD of three separate experiments.

carcinoma cells (Figure 3B). The level of YB-1, both in total and that retained by the *PTP1B* enhancer probe, from extracts of HepG2 cells was higher than in Rat1 cells. Interestingly, this was also reflected in higher levels of PTP1B in HepG2 compared with Rat1 cells (Figure 3B).

We also examined the interaction of YB-1 with the *PTP1B* enhancer in EMSAs. First, we observed that the complex of purified YB-1 and the $-155/-132$ *PTP1B* enhancer probe was supershifted in an EMSA following addition of an anti-YB-1 antibody (Figure 3C). In contrast, although the *PTP1B* enhancer motif displays some features of a recognition site for GATA family-binding proteins (Fukada and Tonks, 2001), antibodies to GATA1 and GATA2 did not affect the mobility of the DNA-protein complex by EMSA. These data suggest that YB-1, rather than a minor component of the purified preparation, is responsible for binding to the functional *PTP1B* enhancer. This was confirmed further by using purified YB-1 from a distinct source, specifically recombinant GST-YB-1 fusion proteins purified following expression in *Escherichia coli* (Figure 3D, left). GST alone did not form a complex with the functional *PTP1B* enhancer probe; however, a complex of purified GST-YB-1 and the $-167/-132$ *PTP1B* enhancer probe was detected by EMSA. Formation of this complex was disrupted by addition of the same cold probe, but not by a probe corresponding to an inactive mutant form of the *PTP1B* enhancer [$-167/-132$ ($\Delta-155/-144$)] (Figure 3D, right). These data illustrate that YB-1 bound directly to the functional *PTP1B* enhancer and, therefore, may be important in control of PTP1B expression *in vivo*.

YB-1 positively regulates PTP1B expression in vivo

To address whether YB-1 is a regulator of PTP1B expression in cells, we established stable Rat1 cell lines expressing an antisense construct specific for YB-1 (Ohga *et al.*, 1998). Approximately 20 clonal lines were established, and three were selected for further analysis, all of which gave similar results. Expression of the antisense YB-1 construct resulted in significant depletion of YB-1 protein, to ~50% of the levels in the parental Rat1 cell controls (Figure 4A, quantitated by densitometry) and did not induce any obvious changes in morphology, cell proliferation or cell death (data not shown). However, depletion of YB-1 protein did lead to an ~70% decrease in the expression of PTP1B, as visualized by immunoblotting with an anti-PTP1B antibody (Figure 4A, quantitated by densitometry). In contrast, we detected no changes in the level of expression of TC-PTP, which is the closest relative of PTP1B, or in the levels of SHP-2 and LAR (Figure 4A), suggesting specificity in the depletion of PTP1B.

As the stable cell lines expressing antisense YB-1 displayed depletion of both YB-1 and PTP1B, we tested the effects of depleting YB-1 protein levels on the activity of the *PTP1B* promoter in two sets of assays. In the first, we examined the activity of *PTP1B* promoter in the context of the pGL3-basic construct, in which the promoter directly controls expression of the luciferase reporter (Figure 4B). In the pGL3-control construct, expression of the luciferase reporter is driven by the SV40 minimal promoter and enhancer elements. The activity of this control was independent of the levels of

YB-1. However, comparison of the activity of the intact *PTP1B* promoter, or the $-167/+145$ construct containing an intact *PTP1B* enhancer, in parental and antisense YB-1-expressing Rat1 cells revealed that depletion of YB-1 led to decreased promoter activity (Figure 4B). Interestingly, the residual activity of the $-151/+145$ promoter construct, in which the *PTP1B* enhancer sequence was disrupted, was not affected by depletion of YB-1 in Rat1 cells (Figure 4B). Similar results were observed when comparison was made with the activity of the pGL3-promoter construct, in which expression of the luciferase reporter is driven by the SV40 minimal promoter (Figure 4C). Comparison with the pGL3-control construct demonstrated that the SV40 enhancer led to an ~3-fold induction of promoter activity. When elements of the *PTP1B* enhancer were linked to the SV40 minimal promoter, we observed that the induction of luciferase activity was similar to that seen with the SV40 enhancer (Figure 4C). Furthermore, the activity of constructs that contained intact enhancer sequences (i.e. $-167/-132$ and $-155/-132$) was inhibited by depletion of YB-1, whereas the residual activity of the constructs in which the enhancer was disrupted [i.e. $-167/-144$ and $-167/-132$ ($\Delta-155/-144$)] was insensitive to depletion of YB-1 (Figure 4C).

These data illustrate that depletion of YB-1 led to both reduction in the level of PTP1B protein and inhibition of *PTP1B* enhancer activity in a cell-based assay. Therefore, we performed the reciprocal experiment and tested the effects of overexpression of YB-1 on the levels of PTP1B. As shown in Figure 5A, overexpression of Flag-tagged YB-1 resulted in a 2-fold increase in the level of PTP1B, consistent with a role for YB-1 as a regulator of the expression of PTP1B.

In order to explore further this potential functional interaction, we compared the levels of expression of YB-1 and PTP1B by PCR of cDNA from the Clontech human cell line MTC panel (Figure 5B, inset). For each cell type, the level of YB-1 and PTP1B cDNA was determined by densitometry of the agarose gel image and was standardized relative to the level of the G3PDH control. For both YB-1 and PTP1B, this ratio in 293 cells was arbitrarily assigned a value of 1 and the data for the other cell lines are presented relative to that (Figure 5B). The data clearly illustrate a correlation between the levels of PTP1B and YB-1 in the cancer cell lines. We also explored this issue further in an animal model of diabetes. Previously, it had been reported that the level of expression and activity of PTP1B is higher in skeletal muscle of non-obese, insulin-resistant type II diabetic Goto-Kakazaki (GK) rats than in non-diabetic, control WKY rats (Dadke *et al.*, 2000). Therefore, we compared the levels of PTP1B and YB-1 by immunoblotting skeletal muscle extracts of overtly diabetic GK and control WKY rats, which were comparable in age and weight. The level of the proteins was quantitated by densitometry of the immunoblot and standardized relative to the actin control. As reported previously, we detected higher levels of PTP1B in skeletal muscle of the diabetic GK rats than in that of the controls. Furthermore, this coincided with higher levels of YB-1 (Figure 5C). Therefore, our data, including the correlation between the levels of YB-1 and PTP1B in models of both cancer and diabetes, are consistent with a functional

relationship in both physiological and pathophysiological conditions.

Depletion of YB-1 in Rat1 cells results in increased sensitivity to insulin and cytokines

Ablation of the *PTP1B* gene in mice, together with structural studies of PTP1B–substrate interactions, have implicated this PTP in the dephosphorylation of the insulin receptor (Tonks and Neel, 2001). Therefore, we tested the effects of antisense-induced depletion of YB-1, and thus PTP1B, on signaling in Rat1 cells. We compared the level of phosphorylation of the insulin receptor β -subunit in parental and antisense YB-1-expressing Rat1 cells and observed no change in the basal level of phosphorylation of the insulin receptor β -subunit. However, hormone-induced tyrosine phosphorylation of the receptor was enhanced by the depletion of YB-1, and the concomitant depletion of PTP1B (Figure 6A). In addition, the phosphorylation of PKB/AKT and Erk MAP kinase, which

represent signaling events downstream of the insulin receptor, were also elevated in antisense YB-1-expressing cells (Figure 6B and C). These effects were counteracted by forced re-expression of PTP1B, which suppressed the enhanced tyrosine phosphorylation of the insulin receptor β -subunit that was induced by antisense YB-1 (Figure 6D). These data suggest that the effects of antisense YB-1 on insulin signaling are mediated by changes in the level of PTP1B, and further emphasize a role for PTP1B in downregulation of hormone-activated insulin receptor.

In light of recent indications that PTP1B also recognizes JAK PTKs as substrates (Myers *et al.*, 2001; Cheng *et al.*, 2002; Zabolotny *et al.*, 2002), we examined signaling responses initiated by stimulation of the cytokine receptor gp130, which is shared by the receptors for the interleukin (IL)-6 family of cytokines (Fukada *et al.*, 1999). For these experiments, we expressed a granulocyte colony-stimulating factor (G-CSF) receptor–gp130 chimera in Rat1 cells and followed signaling events induced by G-CSF. We

Fig. 5. Analysis of the relationship between expression of YB-1 and PTP1B. (A) The effect of overexpression of YB-1 on the levels of PTP1B. Rat1 cells were transiently transfected with 10 μ g of expression plasmid for either Flag-tagged YB-1 (Flag-YB-1) or pCMV-tag empty vector control. Total cell lysate (20 μ g) was subjected to SDS–PAGE followed by immunoblotting with the indicated antibodies to YB-1 and PTP1B, as well as to actin, included as a loading control (left). Densitometric analyses of the gel images, in which expression of YB-1 (black bars) and PTP1B (gray bars) is presented relative to the actin control. (B) Expression pattern of YB-1 and PTP1B in cancer cells. The expression of YB-1 and PTP1B was assessed by PCR of cDNA from the Clontech human cell line MTC panel. The graph represents densitometric analyses of the gel images (inset) to illustrate the ratio of expression over G3PDH control. Black and gray bars indicate the expression level of YB-1 and PTP1B, respectively. (C) Enhanced expression of YB-1 and PTP1B in type II diabetic GK rats. Tissue homogenate (20 μ g) of skeletal muscle from either two individual WKY control rats (C1 and C2) or two individual type II diabetic GK rats (D1 and D2) was subjected to SDS–PAGE followed by immunoblotting with antibodies to YB-1 and PTP1B, or to actin as a loading control. The graph represents densitometric analyses of the gel images to illustrate the ratio of expression over the actin control. Black and gray bars represent YB-1 and PTP1B, respectively.

observed that the activity of JAK1, which is stimulated by dimerization of gp130 (Guschin *et al.*, 1995; Rodig *et al.*, 1998), was enhanced in YB-1-depleted cells compared with controls (Figure 7A); a weak enhancement of JAK2 phosphorylation was also observed, but the levels of this kinase were much lower than those of JAK1 (data not shown). One of the targets of the JAK PTKs is the cytoplasmic segment of the gp130 component of the receptor. We also noted that tyrosine phosphorylation of gp130 was enhanced in cells depleted in YB-1 (Figure 7B). Furthermore, we observed enhanced signaling downstream of the receptor, in that the phosphorylation of STAT3 (Figure 7C) and Erk MAP kinase (Figure 7D) was also enhanced following depletion of YB-1. As observed for insulin signaling, we found that the effects of antisense YB-1 on JAK1 phosphorylation were abrogated by forced re-expression of PTP1B (Figure 7E). These data lend further support to the importance of PTP1B as a mediator of the effects of YB-1 and as a regulator of JAK PTK-mediated signaling responses to cytokines.

Discussion

PTP1B is the prototypic member of the PTP family and has been implicated in the control of tyrosine phosphorylation events induced by growth factors, hormones and cytokines (Tonks and Neel, 2001). In particular, PTP1B plays a crucial role in downregulating signals induced by insulin and leptin (Cook and Unger, 2002; Ukkola and Santaniemi, 2002). Expression of PTP1B is altered in a number of disease conditions associated with aberrant tyrosine phosphorylation. For example, enhanced expression of PTP1B was observed in animal models of diabetes as well as in patients with diabetes and insulin resistance (Ahmad *et al.*, 1997), and polymorphisms that are associated with type II diabetes have been detected in the *PTP1B* gene (Echwald *et al.*, 2002; Mok *et al.*, 2002). Changes in PTP1B expression have also been noted in various cell models. For example, stimulation of L6 skeletal muscle cells with insulin and insulin-like growth factor leads to upregulation of *PTP1B* gene expression, possibly representative of a negative feedback loop (Kenner *et al.*, 1993). Furthermore, the heterotrimeric G-protein G_{i2} enhanced insulin signaling through suppression of PTP1B expression (Tao *et al.*, 2001a). Changes in PTP1B expression have also been associated with various cancers, for example in response to p210 Bcr-Abl, the oncoprotein tyrosine kinase that is the underlying cause of CML (LaMontagne *et al.*, 1998a,b). Overexpression of PTP1B was observed in p185^{neu}/c-erbB-2-associated human breast cancer (Zhai *et al.*, 1993; Wiener *et al.*, 1994b) as well as human ovarian carcinomas (Wiener *et al.*, 1994b), and PTP1B has been implicated as the major PTP capable of dephosphorylating and activating the c-Src PTK (Bjorge *et al.*, 2000). In addition, PTP1B was upregulated in proliferating and migrating smooth muscle cells following balloon catheter-induced acute arterial

Fig. 6. Expression of antisense YB-1 in Rat1 cells led to enhanced sensitivity to insulin. (A) Tyrosine phosphorylation of the β -subunit of the insulin receptor (IR) was enhanced in Rat1 cells expressing antisense YB-1. Control Rat1 cells (Rat1-control) and Rat1 cell clone 20 expressing antisense YB-1 (Rat1-antisense YB-1) were stimulated with insulin (500 nM) for the indicated times. IR β -subunit was then immunoprecipitated from the cell lysate and immunoblotted with either anti-phosphotyrosine or anti-IR β -subunit antibodies. (B and C) Signaling downstream of the insulin receptor was activated in Rat1 cells expressing antisense YB-1. Rat1-control and Rat1-antisense YB-1 cells (clone 20) were stimulated with insulin for the indicated times. Total lysate (20 μ g) was subjected to SDS-PAGE and immunoblotting with anti-phospho-Akt or anti-Akt antibodies (B) and anti-phospho-MAPK (Erk1/2) or anti-MAPK (Erk1/2) antibodies (C). For each of (A–C), the panels on the right-hand side of the figure represent densitometric analyses of the gel images to illustrate the ratio of phosphorylated over total protein. Black squares and open circles indicate Rat1-antisense YB-1 and Rat1-control, respectively. (D) Ectopic expression of PTP1B in Rat1 cells expressing antisense YB-1 suppressed insulin-induced tyrosine phosphorylation of the IR β -subunit. Rat1-antisense YB-1 (clone 20) cells were transiently transfected with expression plasmids (10 μ g each) for either PTP1B (Flint *et al.*, 1997) (PTP1B) or pMT2 empty vector (control). The cells were stimulated with insulin (500 nM) for the indicated times. Total lysate (20 μ g) was subjected to SDS-PAGE followed by immunoblotting with antibodies to PTP1B and actin, included as a loading control. The IR β -subunit was immunoprecipitated from the cell lysate and immunoblotted with either anti-phosphotyrosine or anti-IR β -subunit antibodies. The graph represents densitometric analyses of the gel images to illustrate the ratio of phosphorylated over total protein in the absence (gray bars) or presence (black bars) of ectopically expressed PTP1B.

injury (Wright *et al.*, 2000). Such observations emphasize the importance of understanding the mechanisms by which PTP1B expression is regulated so as to understand fully its role in the regulation of signal transduction in normal and disease conditions.

Currently, there is limited information on the mechanism by which expression of PTP1B is regulated. An insertion in the 3'-untranslated region of the *PTP1B* gene has been mapped and was suggested to increase the stability of PTP1B mRNA and to be associated with

several features of insulin resistance (Di Paola *et al.*, 2002). We have focused on characterizing the regulation of *PTP1B* gene expression at the transcriptional level. We have characterized the *PTP1B* promoter and identified two regulatory elements. The first, termed PRS, controls expression in response to p210 Bcr-Abl through interaction with Egr-1 and the Sp family of C₂H₂ zinc finger transcription factors (Fukada and Tonks, 2001). In this study, we have identified and characterized an enhancer element in the *PTP1B* promoter. We have shown that the transcription factor YB-1 binds to the enhancer and stimulates expression from the *PTP1B* promoter.

Y box-binding proteins comprise two segments. The C-terminal domain is hydrophilic in character and, although it is not strictly conserved between family members, overall it comprises alternating segments of basic and acidic residues. This domain has been implicated in protein-protein interactions. The N-terminal segment has been implicated in transcriptional regulation and contains the nucleic acid-binding domain, which is highly conserved from bacteria to humans (Wolffe, 1994). In YB proteins from vertebrates, this nucleic acid-binding domain displays 43% identity to a cold shock response protein (CS7.4) from *E.coli*. Such a high degree of conservation is reminiscent of the histones and is suggestive of a fundamentally important function. A variety of functional roles have been ascribed to Y box-binding proteins based on their ability to interact with

Fig. 7. Expression of antisense YB-1 in Rat1 cells led to enhanced gp130-mediated signaling. Control Rat1 cells (Rat1-control) and Rat1 cell clone 20, expressing antisense YB-1 (Rat1-antisense-YB-1), were transiently transfected with constructs expressing a chimeric receptor consisting of the extracellular segment of the G-CSF receptor and the transmembrane and cytoplasmic segments of gp130 (Fukada *et al.*, 1996). The cells were stimulated with G-CSF for the indicated times. (A) Activation of JAK1 was enhanced in Rat1 cells expressing antisense YB-1. JAK1 was immunoprecipitated from 1 mg of cell lysate following stimulation with G-CSF, and immunoblotted with either anti-phospho-JAK1 or anti-JAK1 antibodies. (B) Tyrosine phosphorylation of the chimeric receptor was enhanced in Rat1 cells expressing antisense YB-1. The chimeric receptor was immunoprecipitated from 1 mg of stimulated cell lysate with anti-gp130 antibody, then immunoblotted with either anti-phosphotyrosine or anti-gp130 antibodies. (C and D) Signaling downstream of the G-CSF-gp130 receptor was activated in Rat1 cells expressing antisense YB-1. Rat1-control and Rat1-antisense YB-1 cells (clone 20) were stimulated with G-CSF for the indicated times. Total lysate (20 μ g) was subjected to SDS-PAGE and immunoblotting with anti-phospho-STAT3 or anti-STAT3 antibodies (C) and anti-phospho-MAPK (Erk1/2) or anti-MAPK (Erk1/2) antibodies (D). For each of (A–D), the panels on the right-hand side of the figure represent densitometric analyses of the gel images to illustrate the ratio of phosphorylated over total protein. Black squares and open circles indicate Rat1-antisense YB-1 and Rat1-control, respectively. (E) Ectopic expression of PTP1B in Rat1 cells expressing antisense YB-1 suppressed gp130-mediated phosphorylation of JAK1. Rat1-antisense YB-1 (clone 20) cells were transiently transfected with expression plasmids (10 μ g each) for either PTP1B (Flint *et al.*, 1997) (PTP1B) or pMT2 empty vector (control), together with constructs expressing the G-CSFR-gp130 chimeric receptor. The cells were stimulated with G-CSF for the indicated times. Total lysate (20 μ g) was subjected to SDS-PAGE followed by immunoblotting with antibodies to PTP1B and actin, included as a loading control. JAK1 was immunoprecipitated from the cell lysate and immunoblotted with either anti-phospho-JAK1 or anti-JAK1 antibodies. The graph represents densitometric analyses of the gel images to illustrate the ratio of phosphorylated over total protein in the absence (gray bars) or presence (black bars) of ectopically expressed PTP1B.

single- and double-stranded DNA and RNA. They have been implicated in direct transcriptional activation and repression, functioning as transcription factors, as well as in modulating transcription through alteration of promoter conformation, recognizing single-stranded and H-form, triplex DNA. In addition, Y box-binding proteins have been implicated in the control of protein translation through sequestration of mRNA (Wolffe *et al.*, 1992; Wolffe, 1994).

YB-1 was the first Y box-binding protein to be identified, through an expression cloning strategy to find proteins that bound to Y box DNA sequence from the major histocompatibility complex (MHC) class II promoter (Didier *et al.*, 1988). Interestingly, YB-1 has been implicated in the regulation of expression of genes associated with cell proliferation, for example as an activator of transcription of the genes for thymidine kinase, proliferating cell nuclear antigen (PCNA), DNA polymerase α and the EGF receptor (Ladomery and Sommerville, 1995). YB-1 is itself an early response gene that is induced following IL-2 stimulation of cloned T helper lymphocytes (Sabath *et al.*, 1990). YB-1 also functions as a transcriptional repressor, for example in the regulation of *Fas* expression, and thus may regulate survival by coordinating the expression of genes associated with cell proliferation and cell death (Lasham *et al.*, 2000). In addition to its function in transcriptional control, YB-1 is an mRNA-binding protein, for example recognizing a JNK response element in the 5'-untranslated region of IL-2 mRNA and conferring stability to that message during T-cell activation (Chen *et al.*, 2000). As the repertoire of functions of YB-1 has expanded, so has the repertoire of binding sites, such that there now are recognition motifs for YB-1 that have little similarity to the classical Y box, including pyrimidine-rich DNA, interferon response elements and class III gene promoter elements (Wolffe *et al.*, 1992; Wolffe, 1994). This diversity of recognition sites renders it difficult to predict a role for YB-1 in regulating expression of a particular gene from the DNA sequence, as, for example, in this case in which the *PTP1B* enhancer does not contain an obvious Y box motif.

Direct association with the other transcription factors may be important for regulation of gene expression by Y box-binding proteins (Wolffe *et al.*, 1992; Wolffe, 1994; Okamoto *et al.*, 2000). For example, YB-1 coordinates with both AP-2 and p53 to regulate expression of gelatinase A/matrix metalloproteinase 2 (MMP2) (Mertens *et al.*, 2002). Similarly, YB-1 has been shown to associate with p65 RelA, and this interaction augments the association of p65 and inhibits the association of YB-1 with sequences in the human polyomavirus JC virus (JCV) late promoter (Raj *et al.*, 1996). Of particular interest to this study is the observation that there was coordinate regulation of the human multidrug resistance (*MDR*) gene promoter by Y box-binding protein family members and Sp-1, with recognition by both required for optimal promoter activity (Bargou *et al.*, 1997; Sundseth *et al.*, 1997; Hu *et al.*, 2000). The binding of YB-1 to the human immunodeficiency virus type 1 promoter is also regulated by Sp1 (Sawaya *et al.*, 1998). In the case of the *PTP1B* promoter, we have identified two regulatory elements, the enhancer, which recognizes YB-1, and the PRS motif,

which is a recognition site for Sp family transcription factors, and the presence of both is required for optimal promoter activity (Fukada and Tonks, 2001). Whether there are further interactions with additional transcription factors, such as in the minor complex B observed in Figure 2A, remains to be established.

A number of links between YB-1 and human disease conditions have already been reported. For example, high YB-1 expression predicts drug resistance and poor prognosis in human breast cancer (Janz *et al.*, 2002), and the presence of nuclear YB-1 has been linked to progression of non-small cell lung cancer (Shibahara *et al.*, 2001). YB-1 is a regulator of expression of MMP2/gelatinase A which is found in high levels in metastatic tumor cells and correlates with overall metastatic potential (Stetler-Stevenson *et al.*, 1993; Mertens *et al.*, 1998). Interestingly, DU145, a highly aggressive metastatic cancer cell line, possesses relatively high levels of YB-1 and PTP1B (Figure 5B). Our observation that YB-1 directly regulates the level of expression of PTP1B illustrates a novel indirect mechanism for the regulation of tyrosine phosphorylation and suggests a further mechanism through which YB-1 may exert an effect on human disease. This is reinforced by our observation that manipulation of cellular YB-1 levels, by overexpression or expression of an antisense construct, resulted in parallel changes in the levels of PTP1B, coincident with altered signaling in response to insulin (Figure 6). This is entirely consistent with the role of PTP1B as an inhibitor of insulin signaling that has been suggested by the phenotype of *PTP1B* knockout mice (Elchebly *et al.*, 1999; Klamann *et al.*, 2000). In light of our observation that expression of both YB-1 and PTP1B was enhanced in type II diabetic GK rats (Figure 5C), it will be interesting to ascertain whether the expression and/or activation of YB-1 is altered in diabetes and obesity.

Our previous studies also demonstrated that PTP1B may antagonize signaling through JAK family PTKs (Myers *et al.*, 2001). Furthermore, the effects of PTP1B on leptin signaling are exerted primarily at the level of the JAKs (Cheng *et al.*, 2002; Zabolotny *et al.*, 2002). In this study, activation of the chimeric cytokine receptor gp130 resulted primarily in activation of JAK1. Manipulation of the levels of YB-1, and thus PTP1B, also led to altered signaling through the cytokine receptor gp130 coincident with changes in the phosphorylation of JAK1 (Figure 7). These observations provide further support for a role of PTP1B in the regulation of cytokine signaling and in the control of the phosphorylation status of the JAK PTKs.

Members of the PTP family are now recognized as specific regulators of signal transduction processes *in vivo*. There are several aspects to the establishment and maintenance of such specificity. For example, selectivity in PTP-substrate recognition has been characterized at the molecular and structural level (Salmeen *et al.*, 2000). PTP activity is tightly regulated by covalent modifications such as phosphorylation and reversible oxidation (Meng *et al.*, 2002). This current study illustrates a further aspect of the regulation of PTP function, that of control of PTP expression at the transcriptional level. PTP1B is viewed as an exciting novel target for therapeutic intervention in type II diabetes and obesity (Kennedy and Ramachandran, 2000). These data emphasize the fact that changes in the

level, and thus activity, of PTP1B may exert profound effects on signaling in response to insulin and cytokines. It will be interesting to see whether indirect inhibitory effects on PTP1B function at the level of control of expression will provide opportunities for therapeutic development.

Materials and methods

Plasmid construction

Double-stranded DNA sequences from the *PTP1B* promoter were inserted into *MluI*–*XhoI* sites in the pGL3 promoter (Promega) reporter plasmid, in which promoter activity can be detected by expression of firefly luciferase.

The following double-stranded DNA was used for plasmid construction: for –167/–132, 5′-CGCGTACGCGCGCTATTAGATATCTCGCGTGTGGGGCCAC-3′ (sense) and 5′-TCGAGTGGCCCCAGCACC-GCGAGATATCTAATAGCGCGCGTA-3′ (antisense); for –167/–144, 5′-CGCGTACGCGCGCTATTAGATATCTCGCGC-3′ (sense) and 5′-TCGAGCGCGAGATATCTAATAGCGCGCGTA-3′ (antisense); for –167/–156, 5′-CGCGTACGCGCGCTATT-3′ (sense) and 5′-TCGAG-AATAGCGCGCGTA-3′ (antisense); for –155/–132, 5′-CGCGTAGAT-ATCTCGCGGTGCTGGGGCCAC-3′ (sense) and 5′-TCGAGTGGCCCC-CAGCACCAGAGATATCTA-3′ (antisense); for –155/–144, 5′-CGC-GTAGATATCTCGCGC-3′ (sense) and 5′-TCGAGCGCGAGATATCTA-3′ (antisense); for –143/–132, 5′-CGCGTGTGCTGGGGCCAC-3′ (sense) and 5′-TCGAGTGGCCCCAGCACCAGAGATATCTA-3′ (antisense); and for –167/–132 (Δ–155/–144), 5′-CGCGTAACGCGCGCTATTGTGCTGGGGCCAC-3′ (sense) and 5′-TCGAGTGGCCCCAG-CACA-3′ (antisense).

A series of 5′ deletion mutants of the *PTP1B* 5′-flanking region were made by PCR using pGL3-basic:–2k/+145 as a template (Fukada and Tonks, 2001). The 5′ primers used for making deletion mutants were as follows: for –2k/+145, 5′-GGTACCGAGCTCTTACGCGT-3′; for –167/+145, 5′-ACTATAGGGCAGCGTACGCGCGCTATTAGATATCT-3′; and for –151/+145, 5′-ACTATAGGGCAGCGTATCTCGCGGT-GCTGGGGCC-3′. The 3′ primer used for making deletion mutants was 5′-CCCCTCGAGGACGGGCCAGGGCGGCTGCTGCGCTCCTT-3′.

Amplified PCR products were digested by *MluI* and *XhoI* and inserted into the pGL3 promoter (Promega, Madison, WI) reporter plasmid, followed by confirmation by sequencing.

Cell culture

Parental Rat1 fibroblasts and HepG2 human hepatocellular carcinoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Rat1 fibroblasts expressing the antisense YB-1 mRNA were established by stable transfection with the antisense expression construct for YB-1 (AS 0.7 YB-1) (Ohga *et al.*, 1998) and were cultured in DMEM supplemented with 10% FBS in the presence of G418 (Gibco-BRL, Grand Island, NY).

Transfection

Cells were transfected using LipofectAMINE Reagent (Life Technologies, Grand Island, NY) according to the supplier's protocols. Typically, for the reporter assay, 1 μg of the reporter plasmid was used for expression of firefly luciferase, and 1 μg of pRL-TK (Promega, Madison, WI), an expression vector containing cDNA encoding *Renilla* luciferase, was used as an internal control of transfection efficiency. In some experiments, a construct was used that expressed a chimeric receptor consisting of the extracellular domain of the G-CSF receptor with the transmembrane and the cytoplasmic domain of gp130 (Fukada *et al.*, 1996). In this case, 10 μg of the expression plasmid were used for each transient transfection. Cells were incubated with DNA–lipid complex for 24 h, washed with phosphate-buffered saline and assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI), or subjected to other biochemical analyses.

Immunoblotting

Nuclear extract or total cell lysate (20 μg) from each sample was resolved by SDS–PAGE and transferred to a PVDF membrane. Membranes were probed with the indicated antibodies and subjected to enhanced chemiluminescence (ECL; Pharmacia, Uppsala, Sweden). The following antibodies were used. Anti-PTP1B FG6 (LaMontagne *et al.*, 1998a) and anti-TCPTP 1910 (Lorenzen *et al.*, 1995) were as described previously. Anti-YB-1 was a gift from Dr DiCorleto (Stenina *et al.*, 2000). Anti-SHP2

(sc-280), anti-LAR (sc-1119), anti-actin (sc-8432), anti-gp130 (sc-655) and anti-STAT3 (sc-482) were purchased from Santa Cruz (Santa Cruz, CA). Anti-Akt, anti-phospho-Akt, anti-Erk1/2, anti-phospho-Erk1/2 and anti-phospho-Tyr705-STAT3 were purchased from Cell Signaling (Beverly, MA). Anti-JAK1 and anti-insulin receptor β were from Transduction Labs (San Diego, CA) and anti-phospho-JAK1 was from Bio Source (Camarillo, CA). Tyrosine-phosphorylated proteins were detected with antibodies to p-Tyr, either sc-508, purchased from Santa Cruz, or G104, generated in our laboratory (Garton *et al.*, 1997).

Immunoprecipitation

Cells were starved of serum for 6 h, stimulated with 500 nM insulin or 50 ng/ml G-CSF (both from Cal Biochem, La Jolla, CA) for the indicated periods of time, then suspended in 1 ml of lysis buffer [(1% NP-40, 20 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 μg/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na₃VO₄]. Lysates were cleared by centrifugation and mixed with 20 μl of protein A–Sepharose or protein G–Sepharose (Pharmacia, Uppsala, Sweden), and 1 μg of either anti-insulin receptor β, anti-gp130 or anti-JAK1 antibody, followed by incubation at 4°C for 6 h. The immunoprecipitates were eluted with SDS–PAGE loading buffer, separated by SDS–PAGE and transferred to a PVDF membrane (Millipore, Bedford, MA). The membranes were subjected to immunoblotting with individual antibodies as indicated.

DNA pull-down assay

The DNA pull-down assay was performed as described previously (Fukada and Tonks, 2001). Briefly, nuclear extracts were prepared from either parental Rat1 fibroblasts or Rat1-derived cell lines according to the method of Sadowski and Gilman (1993). Nuclear extract was obtained using nuclear extraction buffer [20 mM HEPES pH 7.9, 20 mM NaF, 1 mM Na₃VO₄, 1 mM Na₄P₂O₇, 0.125 μM okadaic acid, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 0.2% NP-40, 420 mM NaCl, 5% glycerol]. Nuclear extract (100 μg) was incubated in a final volume of 1 ml of nuclear extraction buffer containing 100 mM NaCl, together with each biotinylated DNA probe (200 pmol) and 20 μl of streptavidin–Sepharose High Performance (Pharmacia, Uppsala, Sweden) at 4°C for 6 h. The protein–DNA complexes were subjected to SDS–PAGE followed by immunoblotting with anti-YB-1 antibodies. Bound proteins were visualized by ECL (Pharmacia, Uppsala, Sweden).

The biotinylated double-stranded DNA probes used for EMSAs were: for –167/–132, biotin-5′-ACGCGCGCTATTAGATATCTCGCGGTGC-TGGGGCCA-3′ (sense) and biotin-5′-TGGCCCCAGCACCAGCAGATATCTAATAGCGCGCGT-3′ (antisense); for –167/–144, biotin-5′-ACGCGCGCTATTAGATATCTCGCGC-3′ (sense) and biotin-5′-CGCGAGATATCTAATAGCGCGCGT-3′ (antisense); for –155/–132, biotin-5′-AGATATCTCGCGGTGCTGGGGCCA-3′ (sense) and biotin-5′-TGGCCCCAGCACCAGCAGATATCT-3′ (antisense); and for –167/–132 (Δ–155/–144), biotin-5′-ACGCGCGCTATTGTGCTGGG-GCCA-3′ (sense) and biotin-5′-TGGCCCCAGCACAATAGCGCGC-GT-3′ (antisense).

Electrophoretic mobility shift assays

EMSAs were carried out to visualize proteins that bound to sequences from the *PTP1B* enhancer. Double-stranded oligonucleotides were end-labeled using a 5′ end labeling kit (Pharmacia, Uppsala, Sweden), and purified by ProbeQuant™ G-50 Micro columns (Pharmacia, Uppsala, Sweden). The probe (1 × 10⁵ c.p.m.) was incubated with either 5 μg of nuclear extract, 5 μl aliquots from each fraction eluted from SP–Sepharose or the indicated amount of purified GST fusion proteins, for 20 min at 25°C in binding buffer containing 10 mM HEPES–KOH pH 7.8, 50 mM KCl, 1 mM EDTA, 5 mM MgCl₂, 10% glycerol, 5 mM DTT, 0.7 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml pepstatin, 2 μg/ml leupeptin, 1 mM Na₃VO₄, 0.4 μg/ml poly(dI:dC).

The double-stranded DNA probes used for EMSAs and described in Figure 2 were as follows: for –167/–132, 5′-ACGCGCGCTATTAGATATCTCGCGGTGCTGGGGCCA-3′ (sense) and 5′-TGGCCCCAGCACCAGCAGATATCTAATAGCGCGCGT-3′ (antisense); for –167/–144, 5′-ACGCGCGCTATTAGATATCTCGCGC-3′ (sense) and 5′-CGCGAGATATCTAATAGCGCGCGT-3′ (antisense); for –155/–132, 5′-AGATATCTCGCGGTGCTGGGGCCA-3′ (sense) and 5′-TGGCCCCAGCACCAGCAGATATCT-3′ (antisense); and for –167/–132 (Δ–155/–144), 5′-ACGCGCGCTATTGTGCTGGGGCCA-3′ (sense) and 5′-TGGCCCCAGCACAATAGCGCGCGT-3′ (antisense).

The analysis by EMSA of the effects of antibodies on the interaction between YB-1 and the *PTP1B* enhancer probe (Figure 3C) utilized 10 ng of the purified YB-1 preparation and the double-stranded DNA probe

–155/–132 described above. Anti-GATA1 (sc-266X) and anti-GATA2 (sc-9008X) antibodies, purchased from Santa Cruz (Santa Cruz, CA), were included as controls.

Escherichia coli BL21 were transfected with expression plasmid for GST and GST–YB-1 (Izumi *et al.*, 2001), and the recombinant proteins were purified according to Frangioni and Neel (1993). The analysis by EMSA of the interaction between bacterially expressed, GST-tagged YB-1 and the *PTP1B* enhancer probe *in vitro* (Figure 3D) utilized 1 or 10 ng of purified GST or GST–YB-1 protein and the double-stranded DNA probe –167/–132 described above. Unlabeled double-stranded DNA probes –167/–132 and –167/–132 (Δ–155/–144) were tested as competitors of complex formation.

Purification of proteins that bound to the *PTP1B* enhancer sequence

Fractions were assayed by EMSA, using the double-stranded DNA sequence from –167 to –132 of the *PTP1B* promoter as a probe, for the presence of enhancer-binding proteins. To quantify specific binding between proteins and the DNA probe, the EMSA gel was dried and scanned with a BASS 2000 (Fuji) phosphorimager to detect radioactivity in the DNA–protein complex. The specific activity [photostimulated luminescence (PSL)/mm²/μg] was obtained by dividing the PSL of the DNA–protein complex in a defined surface on the gel (PSL/mm²) by the amount of protein (μg) used for EMSA.

Nuclear extracts (~50 mg) were obtained from Rat1 cells, as described above, dialyzed against buffer A (20 mM HEPES pH 7.9, 0.1 M NaCl, 20 mM NaF, 1 mM Na₃VO₄, 1 mM Na₄P₂O₇, 0.125 μM okadaic acid, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 0.2% NP-40, 5% glycerol) and applied to SP Sepharose (20 ml, Pharmacia, Uppsala, Sweden) equilibrated in buffer A. Bound proteins were eluted in a stepwise manner with buffer A containing 0.2, 0.3, 0.4, 0.6 and 0.8 M NaCl. Each fraction was assayed by EMSA for the presence of enhancer-binding proteins. The active fraction, which eluted in 0.8 M NaCl, was dialyzed against buffer A containing 0.2 M NaCl and applied to a double-stranded DNA-conjugated affinity chromatography support. The biotinylated DNA sequence between –167 and –132 bp from the transcription start site, which was used for affinity chromatography, was as follows: biotin-5'-ACGCGCGCTATTAGATATCTCGCGGTGCTGGGGCCA-3' (sense) and biotin-5'-TGGCCCCAGCACCGCGAGATATCTAATAGCGCGCT-3' (antisense). Streptavidin–Sepharose High Performance (2 ml) (Pharmacia, Uppsala, Sweden) was used to conjugate the biotinylated DNA. Bound proteins were eluted in a stepwise manner with buffer A containing 0.2, 0.3, 0.4, 0.6 and 0.8 M NaCl. Each fraction was assayed by EMSA, as above. The active fractions (0.8 M NaCl elution, fractions 1–3, Figure 2B) were concentrated and desalted using Ultrafree-4 Centrifugal Filter Units (Millipore, Bedford, MA) with a 5000 molecular weight cut-off.

The near homogeneous preparation of *PTP1B* enhancer-binding protein was resolved further by SDS–PAGE and visualized using Coomassie Brilliant Blue G–colloidal concentrate (Sigma, St Louis, MO). The major protein band was excised from the gel and subjected to tryptic digestion, then to sequence analysis by tandem mass spectrometry at the Cold Spring Harbor Laboratory Protein Chemistry Shared Resource.

Expression of *PTP1B* and YB-1 in disease models

PCRs were carried out to assess the expression pattern of YB-1 and *PTP1B* in several human cancer cells, using cDNAs from the human cell line MTC™ panel (Clontech, CA). The cDNAs were from the following cancer cell lines; 293 (transformed primary embryonal kidney), SK-OV-3 (malignant ascites fluid from ovary adenocarcinoma), Saos-2 (osteosarcoma), A-431 (epidermoid carcinoma), DU145 (prostate carcinoma metastatic to brain), H1299 (non-small cell lung carcinoma), HeLa (cervical epitheloid carcinoma) and MCF7 (breast adenocarcinoma). PCR was performed according to the manufacturer's protocol, using the following primers: for YB-1, 5'-TTGGGAACAGTAAATGGTTCAAT (sense) and 5'-CTGCTTCTGTCTCTTTGCCATCTT (antisense); and for *PTP1B*, 5'-ATGGAGATGGAAAAGGAGTTCGAG-3' (sense) and 5'-GATATACTCATTCTCTTGATG-3' (antisense).

Skeletal muscle from WYK control and insulin-resistant, type II diabetic GK rats, which were used in a previous study (Dadke *et al.*, 2000), were generously provided by Dr Najma Begum (Diabetes Research Laboratory, Winthrop Hospital and SUNY Stony Brook, Mineola, NY). The GK rats (D1 and D2) displayed typical characteristics of type II diabetes, such as high blood sugar, and their average body weight was 20 g heavier than that of the WKY controls (C1 and C2). The

skeletal muscle samples (~2 g) were homogenized in 50 mM Tris–HCl pH 8.0, 137 mM NaCl, 10% glycerol, 1% NP-40, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 0.5 mM EDTA. The homogenates were clarified by centrifugation and the levels of *PTP1B* and YB-1 were assessed by immunoblotting.

Acknowledgements

We are grateful to Dr R.Kobayashi for protein microsequencing, Dr DiCorleto for YB-1 antibody, Drs M.Kuwano and T.Uchiumi for the YB-1 antisense and Flag-YB-1 plasmids, Dr K.Kohno for the GST–YB-1 plasmid, Dr N.Begum for skeletal muscle of type II diabetic GK and control WKY rats, and Dr Bill Tansay, as well as our colleagues in our laboratory, for critical reading of the manuscript. This work was supported by a grant from the National Institutes of Health (R37-CA53840) to N.K.T. T.F. was supported by the Naito Foundation and a Japan Society for the Promotion of Science Postdoctoral Fellowship for Research Abroad.

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*Received July 29, 2002; revised October 28, 2002;
accepted December 6, 2002*