TYK2 and JAK2 Are Substrates of Protein-tyrosine Phosphatase 1B*

Received for publication, October 9, 2001, and in revised form, October 31, 2001
Published, JBC Papers in Press, November 1, 2001, DOI 10.1074/jbc.C100583200

Michael P. Myers‡§, Jannik N. Andersen‡§, Alan Cheng**, Michel L. Tremblay**, Curt M. Horvath‡‡, Jean-Patrick Parisien‡‡, Annette Salmeen§§, David Barford†¶¶, and Nicholas K. Tonks‡‡‡

From the ‡Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724, the **McGill Cancer Center, McGill University, Montreal, Quebec H3G 1Y6, Canada, the ‡‡Immunobiology Center, Mount Sinai School of Medicine, New York, New York 10029, the §§Laboratory of Molecular Biophysics, University of Oxford, South Parks Road, Oxford OX2 3QU, United Kingdom, and the ¶¶Section of Structural Biology, Institute for Cancer Research, London SW3 6JB, United Kingdom

The reversible tyrosine phosphorylation of proteins, modulated by the coordinated actions of protein-tyrosine kinases and protein-tyrosine phosphatases (PTPs), regulates the cellular response to a wide variety of stimuli. It is established that protein kinases possess discrete sets of substrates and that substrate recognition is often dictated by the presence of consensus phosphorylation sites. Here, we have extended this concept to the PTPs and demonstrated that (E/D)-pY-pY-(R/K) is a consensus substrate recognition motif for PTP1B. We have shown that JAK2 and TYK2 are substrates of PTP1B and that the substrate recognition site within these kinases is similar to the site of dephosphorylation previously identified within the insulin receptor. A substrate-trapping mutant of PTP1B formed a stable interaction with JAK2 and TYK2 in response to interferon stimulation. Expression of wild type or substrate-trapping mutant PTP1B inhibited interferon-dependent transcriptional activation. Finally, mouse embryo fibroblasts deficient in PTP1B displayed subtle changes in tyrosine phosphorylation, including hyperphosphorylation of JAK2. The closely related JAK family member, JAK1, which does not match the consensus dephosphorylation site, was not recognized as a substrate. These data illustrate that PTP1B may be an important physiological regulator of cytokine signaling and that it may be possible to derive consensus substrate recognition motifs for other members of the PTP family, which may then be used to predict novel physiological substrates.

*This work was supported by Grant CA53840 and Grant P30CA45508 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ These authors contributed equally to this work.

‡ Supported by a fellowship from the V Foundation.

¶¶ To whom correspondence should be addressed: Cold Spring Harbor Laboratory, Demerec Bldg., 1 Bungtown, Cold Spring Harbor, NY 11724-2208. Tel.: 516-367-8846; E-mail: tonks@cshl.org.

The major regulatory mechanism that underlies cellular responses to external stimuli is reversible protein phosphorylation. For example, most growth factors and certain hormones exert their effects through the activation of protein-tyrosine kinases (PTKs). Furthermore, members of the protein-tyrosine phosphatase (PTP) family cooperate with PTKs to fine-tune the responses to extracellular stimuli. In fact, PTPs act not only to attenuate PTK-dependent signaling, but also in cooperation with PTKs to promote signaling (1). The identification of new PTPs has occurred at a rapid rate, with one of the remaining challenges being to identify their physiological substrates.

Before their initial characterization and identification, it was expected that there would be a small number of highly active, widely expressed PTPs, which would exhibit broad substrate specificity. In contrast, it has become clear that the PTP family is large and diverse, and that the enzymes may exhibit exquisite substrate specificity in vivo (2–4). Members of the PTP family are defined by the presence of a conserved signature motif, (I/V)HC where (I/V)HC is a consensus substrate recognition motif for PTP1B. We have shown that JAK2 and TYK2 are substrates of PTP1B and that the substrate recognition site within these kinases is similar to the site of dephosphorylation previously identified within the insulin receptor. A substrate-trapping mutant of PTP1B formed a stable interaction with JAK2 and TYK2 in response to interferon stimulation. Expression of wild type or substrate-trapping mutant PTP1B inhibited interferon-dependent transcriptional activation. Finally, mouse embryo fibroblasts deficient in PTP1B displayed subtle changes in tyrosine phosphorylation, including hyperphosphorylation of JAK2. The closely related JAK family member, JAK1, which does not match the consensus dephosphorylation site, was not recognized as a substrate. These data illustrate that PTP1B may be an important physiological regulator of cytokine signaling and that it may be possible to derive consensus substrate recognition motifs for other members of the PTP family, which may then be used to predict novel physiological substrates.

1 The abbreviations used are: PTK, protein-tyrosine kinase; PTP, protein-tyrosine phosphatase; IR, insulin receptor; IFN, interferon; MEF, mouse embryonic fibroblast.
A Consensus PTP1B Substrate Recognition Motif

and a basic residue, which flank the tandem Tyr(P) motif on the N- and C-terminal side, respectively, form unique interactions with the surface of PTP1B, contributing to the observed high affinity of PTP1B for tandem Tyr(P)-containing peptides relative to their monophosphorylated counterparts (13). The presence of a second aryl phosphate binding site in PTP1B has also been reported by others (14), and it has been shown that short peptides containing vicinal nonhydrolyzable analogs of Tyr(P) are potent inhibitors of PTP1B (15). With the exception of the closely related TCPTP, other PTPs do not appear to possess this second Tyr(P) recognition site, suggesting that PTP1B and TCPTP are likely to recognize tandem Tyr(P) residues. Interestingly, it was shown previously that TCPTP preferentially recognizes the adaptor protein Shc as a substrate when it is phosphorylated on the tandem Tyr(P) residues 239 and 240 (16).

It has been established that assembly of the components of signal transduction pathways and propagation of the signal in response to a particular stimulus requires sequence-specific protein-protein recognition. For example, this occurs not only at the level of substrate phosphorylation by protein kinases (17), but also in protein-protein interactions directed by signaling modules such as SH2 domains (18). In this current study, we have investigated whether the motif (E/D)-pY-pY-(R/K), which was identified previously as being important for the interaction of PTP1B with the activation segment of the IR (13), could be used to predict further novel physiological substrates for PTP1B. Upon searching sequence data bases we noted the presence of this motif in the PTKs TYK2 and JAK2, which are essential mediators of cytokine signaling that are required to activate the STAT family of transcription factors (19, 20). In this study, we have examined the interaction of substrate-trapping PTP1B mutants with these PTKs, tested the effects of PTP1B on STAT-dependent reporters, and looked at the status of tyrosine phosphorylation in PTP1B-deficient fibroblasts. Our data revealed that TYK2 and JAK2 are bona fide substrates of PTP1B and demonstrate that PTP1B plays a role in the negative regulation of cytokine signaling. Importantly this study illustrates that it may be possible to derive consensus substrate recognition motifs for members of the PTP family, which can be used to predict novel substrates and provide insights into physiological function.

EXPERIMENTAL PROCEDURES

Tissue Culture and Transfection—All cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics. HEK293 cells were transfected via calcium phosphate co-precipitation with 20 μg of DNA/10-cm dish. The calcium phosphate/DNA co-precipitate was removed 16 h after addition, and the cells were returned to growth medium for 36 h before harvesting. Transfection efficiency was determined by inclusion of a GFP expression plasmid (1 μg) in all transfections.

Immunoblotting and Immunoprecipitation—Cell lysate, immunoblotting, and substrate trapping experiments were performed as described in Ref. 2. Protein levels were determined by the method of Bradford, using bovine serum albumin as a standard, and equal protein was loaded in each lane. TYK2 antibodies were obtained from Cell Signaling Technology. JAK1 and JAK2 antibodies were obtained from Upstate Biotechnology. The phospho-specific antibodies (Tyr(P)-TYK21054/1055 and Tyr(P)-JAK21007/1008) were from BIOSOURCE International (Camarillo, CA), and the phosphospecific STAT antibodies (Tyr(P)-STAT1 and Tyr(P)-STAT3) were from Cell Signaling Technology.

IFN-responsive Reporter Gene Assays—For luciferase reporter gene assays, cells were transfected with a β-galactosidase expression plasmid (CMV-β-gal), as a control for transfection efficiency, the reporter gene, and either empty vector or cDNA expression plasmids for PTP1B, as indicated. The reporter constructs contained a TATA box and the gene, and either empty vector or cDNA expression plasmids for PTP1B, JAK2, and the phosphospecific STAT antibodies (Tyr(P)-STAT1 and Tyr(P)-STAT3) were from Cell Signaling Technology.

IFN-α, IFN-γ, and IFN-β responses were determined in HEK293 cells. Following a 15-min stimulation with IFN-α, both JAK1 and JAK2 were phosphorylated on tyrosyl residues. However, we were only able to detect an interaction between the substrate trapping mutant form of PTP1B and endogenous TYK2 (Fig. 1B), but not with JAK1 (Fig. 1C). Similarly, we observed that in response to IFN-γ, both JAK1 and JAK2 were phosphorylated, and endogenous JAK2 was recovered in a complex with PTP1B (Fig. 1B). However, no complex formation with JAK1 was detected (Fig. 1C). It is important to note that the interaction between the JAK family members and the PTP1B-trapping mutant was ligand-dependent, indicating that the interaction is dependent upon the activation and tyrosine phosphorylation of TYK2 and JAK2 (Fig. 1B). Furthermore, we did not observe these interactions when either wild type PTP1B (Fig. 1, B and C) or a His-tagged mutant form of TYK2 (TYK2-Y1054/1055F), which lacks the tandem phosphorylation sites within the consensus motif, were co-expressed with the PTP1B-DA-trapping mutant (data not shown). These observations suggest that PTP1B recognizes TYK2 and JAK2 as substrates in this context. However, although JAK1 was activated and tyrosine-phosphorylated in this system (data not shown), no interaction with PTP1B was detected (Fig. 1C), indicating selectivity of PTP1B toward TYK2 and JAK2, the two family members that match the proposed consensus substrate recognition motif. Specificity in the interaction was further emphasized by the fact that upon immunoblot-
result in global changes in the number of tyrosine-phosphorylated proteins. Therefore, we compared the extent of tyrosine phosphorylation in PTP1B/+ and PTP1B−/− mouse embryo fibroblasts (MEFs) (32) under basal conditions and following stimulation by insulin, IFN-α and IFN-γ. Strikingly, we observed that the overall profile of tyrosine phosphorylated proteins was remarkably similar in the two cell lines (Fig. 3A). The only major difference observed was a decrease in phosphorylation of a protein of ~130 kDa in the PTP1B−/− cells, which is consistent with the finding that genetic disruption of PTP1B results in a decrease in integrin-dependent signaling (32). In addition, we observed that insulin-induced tyrosine phosphorylation was potentiated in PTP1B−/− MEFs compared with their PTP1B+/+ counterparts (Fig. 3A), consistent with the identification of PTP1B as one of the primary PTPs that regulates the phosphorylation status of the insulin receptor (33, 34). Furthermore, using a phosphospecific antibody to the activation loop of JAK2, we observed that the PTP1B consensus recognition motif within JAK2 was hyperphosphorylated in the PTP1B-deficient cells relative to PTP1B+/+ controls (Fig. 3B). To study the significance of increased JAK2 phosphorylation on downstream signaling, we examined the activation status of two effectors of interferon signaling, STAT1 and STAT3, for which phospho-specific antibodies are commercially available. The STAT3 blots (Fig. 3C, top panel) revealed increased interferon signaling in PTP1B−/− cells relative to PTP1B+/+ controls, in each condition tested. Furthermore, following IFN-α stimulation we observed enhanced phosphorylation of STAT1 in PTP1B-deficient MEFs compared with the wild type controls. However, this difference in STAT1 phosphorylation was not apparent following IFN-γ treatment, consistent with phosphorylation of this transcription factor primarily by JAK1 or another kinase that is not a direct target of PTP1B. These data, which are consistent with those obtained using substrate trapping mutant forms of the phosphatase (Fig. 1), further emphasize that PTP1B exhibits restricted substrate specificity in vivo.

Although one aspect of this specificity may arise from the restricted subcellular localization of PTP1B in the endoplasmic reticulum (8), our data are also consistent with specificity intrinsic to the catalytic domain. The latter aspect of specificity is now understood in structural terms as arising from the presence of two Tyr(P)-binding pockets, the active site and a second, non-catalytic Tyr(P)-binding site that is not connected to the active site by a short channel (13). In most other PTPs, including PTP-MEG2, access to the second Tyr(P)-binding site is occluded due to the presence of bulky hydrophobic residues at the position equivalent to Gly259 in PTP1B (35). Based on amino acid sequence alignments of PTP catalytic domains, only TCPTP is predicted also to have this second Tyr(P)-binding site (5). The present study also highlights the importance of the acidic and basic residues, which flank the vicinal Tyr(P) residues and interact with surface...
features that are unique to PTP1B, in substrate recognition (13). Although this motif is important for optimal substrate recognition, its presence is not essential for dephosphorylation of a protein by PTP1B. Interestingly, there are reports that PTP1B can accommodate substrates that do not possess tandem Tyr(P) residues (2). It is possible that even in these cases the second Tyr(P)-binding site may still be engaged, with a water molecule forming a bridge between the substrate and Arg24 in the second Tyr(P)-binding pocket (36).

Two important conclusions can be drawn from this study. First, using the structure of a complex between PTP1B and a phosphorylated peptide derived from the activation loop of the IR, we noted previously that the presence of vicinal Tyr(P) residues in a potential substrate enhanced the affinity of its interaction with PTP1B. Furthermore, we had identified the sequence (D/E)-pY-pY-(R/K) as a potential consensus substrate recognition motif (13). This current study illustrates that this sequence motif can be used successfully to identify additional physiological protein substrates for PTP1B. Although this motif may represent a minimal sequence motif, it is possible that additional contacts between PTP1B and its substrates may contribute to affinity and specificity. Such additional determinants of substrate recognition remain to be clarified. Nevertheless, the definition of consensus phosphorylation sites has proven to be an important tool in signal transduction research for the identification of substrates for protein kinases. This study demonstrates for the first time that this concept may also be applicable to protein phosphatases, such as PTP1B, and that consensus recognition sites may also exist for other PTPs. Perhaps it may be possible in the future to use this approach to expand the repertoire of physiological substrates of other members of the PTP family of enzymes.

Second, we have identified PTP1B as a potential regulator of cytokine-dependent signaling. There has been another report indicating that PTP1B may exert an inhibitory effect on JAK-STAT signaling; however, in that case it was suggested that the primary target of PTP1B was STAT-5 (37). The reason for the discrepancy between that report and our current study remains to be ascertained. Nevertheless, when one considers that the major site of regulatory STAT dephosphorylation is believed to be in the nucleus (38), and PTP1B is known to be cytoplasmic (8), its contribution to the physiological regulation of STAT dephosphorylation will require further clarification. Furthermore, the process of STAT dephosphorylation remains poorly understood and, again in contrast to (37), other PTPs such as SHP-2 (29) have also been implicated in the process. In this study, we have shown that PTP1B recognizes TYK2 and JAK2, but not JAK1, and can modulate signaling responses to IFN-α and IFN-γ. One of the striking aspects of the phenotype of PTP1B-deficient mice is its resistance to weight gain when fed a high fat diet (33, 34). Some of these effects may arise from defects in signaling in response to the satiety hormone leptin in the hypothalamus of PTP1B-deficient mice. Our data, demonstrating recognition of JAK2 and TYK2 as substrates by PTP1B, suggest that the PTP may normally function as a negative regulator of leptin signaling in the brain. Thus it is possible that dysfunctional regulation of members of the JAK subfamily of PTKs may contribute to the resistance to weight gain displayed by PTP1B-deficient mice.