The EMBO Journal vol.15 no.1 pp.135–142, 1996

PTP1D is a positive regulator of the prolactin signal leading to β-casein promoter activation

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Stimulation of the prolactin receptor (PRLR), a member of the cytokine/growth hormone receptor family, results in activation of the associated Jak2 tyrosine kinase and downstream signaling pathways. We report that PTP1D, a cytoplasmic protein tyrosine phosphatase containing two Src homology 2 (SH2) domains, physically associates with the PRLR–Jak2 complex and is tyrosine-phosphorylated upon stimulation with prolactin. The formation of the trimeric PRLR–Jak2–PTP1D complex is critical for transmission of a lactogenic signal, while PTP1D phosphorylation is necessary, but not sufficient. The dominant negative inhibitory effect of a phosphatase-deficient mutant on expression of a β-casein promoter-controlled reporter gene is evidence for an essential role of fully functional PTP1D in the regulation of milk protein gene transcription.

Keywords: Jak2/prolactin/prolactin receptor/protein tyrosine phosphatase 1D/signal transduction

Introduction

The polypeptide hormone prolactin (PRL) regulates a variety of biological functions in diverse cell types, including the production of milk proteins in the lactating mammary gland, the development of reproductive organs, immune defense functions and certain behaviors (for a review see Doppler, 1994). The receptor for prolactin (PRLR) is a member of the hematopoietic receptor superfamily (Bazan, 1989; Kelly *et al.*, 1993). Recent findings indicate that for this receptor type ligand binding results in activation of cytoplasmic tyrosine kinases of the Jak/Tyk and Src type families (reviewed in Ihle *et al.*, 1994). PRLR cDNA has been cloned from different tissues and species (Kelly *et al.*, 1993). Two different forms have been identified in rat: a short form of 291 amino acids (Boutin *et al.*, 1988) and a long form of 591 amino acids (Shirota *et al.*, 1990). These result from alternative splicing of a single gene (Shirota *et al.*, 1990). A third form of PRLR, characterized in the rat lymphoma cell line Nb2 (Ali *et al.*, 1991), is identical to PRLR long form with the exception of a missing segment of 198 amino acids found in the cytoplasmic domain. Functional analyses of these receptors have shown that only the long and the Nb2 forms are capable of transmitting signals that activate PRL-responsive genes, such as β-casein and interferon regulatory factor-1 (IRF-1), while the short form fails to mediate the PRL message in these systems (Lesueur *et al.*, 1991; Ali *et al.*, 1992; O'Neal and Yu-Lee, 1994).

The molecular mechanism of PRL action has been studied primarily in the PRL-dependent Nb2 cell line (Gout *et al.*, 1980). These studies have demonstrated that, similarly to other cytokines, PRL stimulation of responsive cells leads to activation and tyrosine phosphorylation of the associated tyrosine kinase Jak2, the receptor itself and other cellular proteins (Rui *et al.*, 1992, 1994; Campbell *et al.*, 1994; Lebrun *et al.*, 1994). Moreover, other investigators have reported activation of the Jak1 kinase in response to PRL in BaF3 cells stably overexpressing PRLR (Dusanter-Fourt *et al.*, 1994). Mutational analysis of PRLR demonstrated a correlation between the ability of the receptor to transmit a mitogenic signal and association and activation of the Jak2 kinase (Da Silva *et al.*, 1994). Furthermore, we have recently shown that activation of milk protein gene transcription by PRL requires both tyrosine phosphorylation of Jak2 and of the C-terminal part of the receptor (Lebrun *et al.*, 1995a) and we identified a single tyrosine residue on PRLR that is necessary for this biological signal (Lebrun *et al.*, 1995b). The involvement of Jak2 kinase in the PRLR signaling pathway is further demonstrated by the recent cloning of the mammary transcription factor MGF (Wakao *et al.*, 1994), a member of a family of SH2-containing DNA binding proteins of 85–95 kDa that have been termed 'signal transducers and activators of transcription' (Stat) molecules (reviewed in Ihle *et al.*, 1994). MGF has been shown to undergo tyrosine phosphorylation and acquire DNA binding ability in response to PRL treatment (Gouilleux *et al.*, 1994). These and other observations clearly support a major role of protein tyrosine phosphorylation in PRLR-mediated signaling.

Little is known about the function of PTPs in the signaling process initiated by receptors of the cytokine/GH/PRL receptor family. Among the PTPs which have been categorized into membrane-associated and cytosolic enzymes (Charbonneau and Tonks, 1992) certain cytoplasmic enzymes contain two SH2 domains at their N-terminus and a single catalytic domain. In mammals two members have been characterized: PTP1C (Shen *et al.*, 1991) [also known as SH-PTP1 (Plutzky *et al.*, 1992), HCP (Yi *et al.*, 1992) and SHP (Matthews *et al.*, 1992)] and PTP1D (Vogel *et al.*, 1993) [also called SH-PTP2 (Freeman *et al.*, 1992), SH-PTP3 (Adachi *et al.*, 1992), PTP2C (Ahmad *et al.*, 1993) and Syp (Feng *et al.*, 1993)]. While it has been postulated that PTPs function as negative

regulators of signals generated by protein tyrosine kinases, several recent reports indicate that they may in fact be positive signal transmitters. In mammalian cells PTP1D was shown to undergo tyrosine phosphorylation in response to growth factor stimulation (Vogel *et al.*, 1993; Feng *et al.*, 1993) and to associate physically with activated growth factor receptors, such as those for platelet-derived growth factor (PDGF), epidermal growth factor (EGF) (Kazlauskas *et al.*, 1993; Lechleider *et al.*, 1993a; Li *et al.*, 1994) and insulin (Kharitonenkov *et al.*, 1995). These interactions are mediated by the SH2 domains of the phosphatase and were shown to have modulatory effects on the catalytic activity of PTP1D (Vogel *et al.*, 1993; Lechleider *et al.*, 1993b; Case *et al.*, 1994; Sugimoto *et al.*, 1994). In analogy to the role of the *Drosophila corkscrew* (*csw*) gene product, a PTP1D homolog (Perkins *et al.*, 1992), a positive role for PTP1D in growth factor receptor signaling (Bennett *et al.*, 1994; Tauchi *et al.*, 1994; Xiao *et al.*, 1994) as well as in mediating insulin responses (Milarski and Saltiel, 1994; Noguchi *et al.*, 1994; Yamauchi *et al.*, 1995) has been suggested. In both of these systems, however, the mechanism by which PTP1D mediates its positive function in signal transmission remains unclear.

We examine here the role of PTP1D in PRLR signaling. Our data indicate that PTP1D is tyrosine-phosphorylated in response to PRL treatment and physically associates with the PRLR–Jak2 complex. We also show that phosphorylation of PTP1D is dependent on proper assembly of the PRLR–Jak2 complex and demonstrate its requirement for regulation of milk protein gene transcription.

Results

Prolactin stimulates PTP1D tyrosine phosphorylation

We used the pre-T rat lymphoma cell line Nb2, a widely used model system for the study of the PRL signal, to examine the role of PTP1D. Lysates from Nb2 cells stimulated with prolactin were immunoprecipitated with an anti-PTP1D antibody (described in Materials and methods). Immune complexes were run on SDS–PAGE, transferred to nitrocellulose and analyzed with an anti-phosphotyrosine antibody. As shown in Figure 1A, a 72 kDa protein was highly phosphorylated upon PRL stimulation. In addition, less intensely phosphorylated proteins of 110, 125, 130 and 145 kDa were found co-precipitating with PTP1D. The major phosphorylated protein of 72 kDa reacted with an anti-PTP1D monoclonal antibody (Figure 1B) and migrated as a doublet, suggesting the existence of differentially phosphorylated forms of PTP1D as a result of PRL interaction with its receptor in Nb2 cells.

Previous reports have demonstrated tyrosine phosphorylation of PTP1D upon association with RTKs (Feng *et al.*, 1993; Vogel *et al.*, 1993) and it is well established that PRLR, lacking an intrinsic tyrosine kinase function, associates with the cytosolic tyrosine kinase Jak2 for the purpose of signal generation and transmission (Campbell *et al.*, 1994; Lebrun *et al.*, 1994; Rui *et al.*, 1994). To examine a possible role of PTP1D in the PRL signal downstream of Jak2 we examined the presence of this kinase in immunoprecipitates of PTP1D. As shown in Figure 1C, immunodetection with an anti-Jak2 antibody in fact identified the phosphoprotein of 130 kDa as representing Jak2. This band was not seen when pre-immune serum was used for immunoprecipitations (data not shown). These results also suggested constitutive association between Jak2 and PTP1D, as reproducibly there was no significant change in the amount of associated Jak2 protein, yet there was an increase in its phosphotyrosine content following PRL stimulation. Physical association between Jak2 and PTP1D was also observed in Jak2 immunoprecipitates and the presence of an active tyrosine kinase was further demonstrated by an immune complex kinase assay in anti-PTP1D precipitates (data not shown). It therefore appears that PRL stimulation results in receptor dimerization and activation of the associated kinase Jak2, leading in turn to tyrosine phosphorylation of PTP1D.

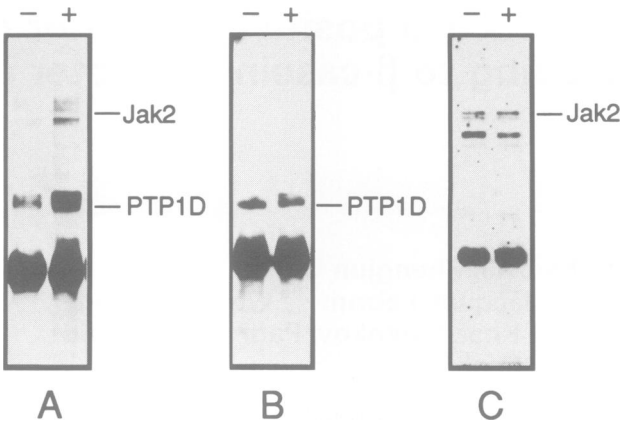

Fig. 1. PTP1D is tyrosine-phosphorylated in response to prolactin stimulation. Nb2 cells (20×10^6) were left unstimulated (–) or stimulated (+) for 10 min with oPRL (10^{-7} M). Cells were solubilized and PTP1D was immunoprecipitated using anti-PTP1D polyclonal antibody (Vogel *et al.*, 1993). Bound proteins were separated by SDS–PAGE, transferred to nitrocellulose and immunodetection was carried out. (**A**) Membrane probed with a monoclonal antibody 4G10 to phosphotyrosine. (**B**) The same membrane as in (A) stripped and immunodetected with a monoclonal antibody to PTP1D. (**C**) Membrane detected with an anti-Jak2 polyclonal specific antiserum. In (C) the band detected at ~120 kDa represents a non-specific band that is usually observed with the anti-Jak2 antibody used in this study.

To characterize the pattern of tyrosine phosphorylation of PTP1D following PRL stimulation we immunoprecipitated PTP1D from lysates of Nb2 cells exposed to PRL for different times. Proteins were separated by SDS–PAGE and anti-phosphotyrosine immunoblot analysis was performed. As indicated in Figure 2A, upon PRL addition to the cells PTP1D rapidly became tyrosine-phosphorylated and its phosphotyrosine content remained elevated during the time period examined, indicating a functional interaction of this phosphatase with PRLR–Jak2 complex.

Jak2 kinase activity is required for PTP1D tyrosine phosphorylation

Studies in a transient overexpression system were carried out to investigate further the interaction of PTP1D with the PRLR–Jak2 complex. For this purpose we employed the human embryonic kidney fibroblast cell line 293 stably overexpressing Jak2 (Lebrun *et al.*, 1995a,b). Cells were transiently co-transfected with expression plasmids carrying the cDNA for PRLR long form (Shirota *et al.*, 1990)

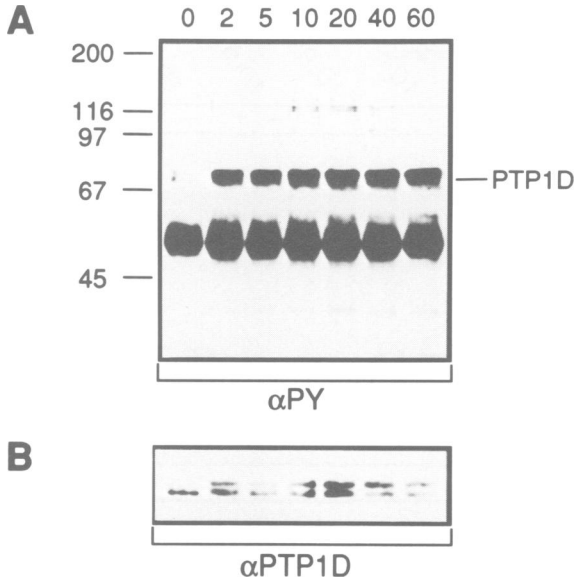

Fig. 2. Time response of PTP1D tyrosine phosphorylation following PRL treatment. Nb2 cells (20×10^6) were stimulated with oPRL (10^{-7} M) for the indicated times. PTP1D was immunoprecipitated from cell lysates. Bound proteins were subjected to SDS–PAGE and transferred to nitrocellulose membranes. (**A**) Immunodetection was carried out with the monoclonal anti-phosphotyrosine antibody 4G10 (UBI). (**B**) The membrane was stripped and reblotted with monoclonal antibody to PTP1D.

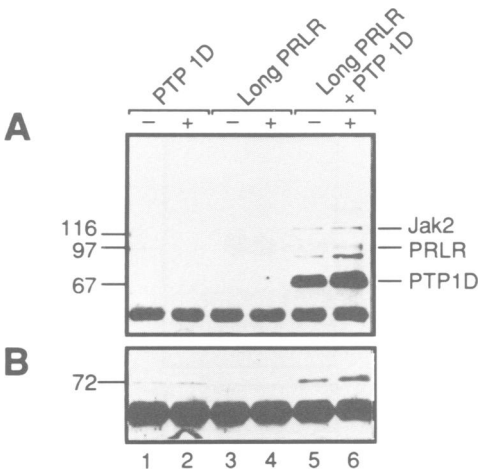

Fig. 3. Activation of the tyrosine kinase Jak2 is required for PRL-dependent tyrosine phosphorylation of PTP1D. (**A**) Human 293-LA cells (Lebrun *et al.*, 1995) were transfected with either the human PTP1D expression plasmid (Vogel *et al.*, 1993) (lanes 1 and 2), PRLR long form expression vector (Shirota *et al.*, 1990) (lanes 3 and 4) or a combination of the two plasmids (lanes 5 and 6). Cells were stimulated (+) with oPRL (10^{-7} M) for 10 min or left untreated (–). Cells were lysed and immunoprecipitation of PTP1D performed. Proteins were separated by SDS–PAGE and transferred onto a nitrocellulose membrane. (**A**) Membrane probed with monoclonal antibody 4G10 to phosphotyrosine. (**B**) The same membrane stripped and reprobed with anti-serum against PTP1D (Vogel *et al.*, 1993). The positions of PTP1D, PRLR and Jak2 are indicated.

and the cDNA encoding PTP1D (Vogel *et al.*, 1993). As shown in Figure 3A and B, tyrosine phosphorylation of immunoprecipitated PTP1D was observed when PTP1D and PRLR were co-expressed in 293-LA cells (lanes 5 and 6). These anti-PTP1D precipitates also contained phosphorylated PRLR (Lebrun, 1995a,b) and Jak2. The identity of the latter was confirmed in parallel immunoprecipitations of the same lysates with anti-Jak2 polyclonal antibody and detection with anti-phosphotyrosine antibody (data not shown). In addition, phosphorylation of endogenous PTP1D was observed when PRLR long form was overexpressed in 293-LA cells (data not shown). In contrast, transfection of a PTP1D expression plasmid alone in this system did not result in its phosphorylation on tyrosines (Figure 3A, lanes 1 and 2) in spite of detectable expression levels, as demonstrated by reprobing the immunoblot with anti-PTP1D polyclonal antibody (Figure 3B). Our results indicated that PTP1D became tyrosine-phosphorylated in response to PRL and that this effect involved physical association with the PRLR–Jak2 complex. Furthermore, since previous reports had demonstrated tight regulation of Jak2 kinase activity by erythropoietin or interleukin-3 stimulation *in vivo* (Silvennoinen *et al.*, 1993; Miura *et al.*, 1994), a direct functional connection to the observed phosphorylation of PTP1D was likely.

Recently it has been demonstrated that a cytoplasmic proline-rich domain conserved among different members of the cytokine/GH/PRL receptor family is needed for Jak2 association and consequent kinase activation (Da Silva *et al.*, 1994; Quelle *et al.*, 1994; Sotiropoulos *et al.*, 1994; VanderKuur *et al.*, 1994; Lebrun *et al.*, 1995a). Therefore, to further support our conclusion that Jak2 kinase activity is necessary for PTP1D tyrosine phosphorylation we transiently co-transfected plasmids encoding PTP1D with two different forms and one mutant of PRLR into 293 cells stably overexpressing Jak2. As shown in Figure 4A, while those forms of PRLR that were capable of associating and activating Jak2 (Lebrun *et al.*, 1995a) were also competent in mediating tyrosine phosphorylation of PTP1D (lanes 2 and 4), the mutant Δ243–267, lacking the association domain with Jak2 (Lebrun *et al.*, 1995a), failed to mediate this reaction (Figure 4A, lanes 5 and 6). The apparent lack of a stimulatory effect of PRL on PTP1D phosphorylation in these experiments was likely due to overexpression of PRLR, as observed previously (Lebrun *et al.*, 1995a), resulting in constitutive receptor/Jak2 activation and constitutive phosphorylation of PTP1D. Taken together, our data demonstrate that PTP1D phosphorylation on tyrosine residues is dependent on the formation of a functional PRLR–Jak2 complex.

Phosphorylation of PTP1D is regulated by its SH2 and catalytic domains

A characteristic structural feature of PTP1D are the two SH2 domains located in the N-terminal portion of the molecule. These domains were shown to mediate association of the phosphatase with activated RTKs and phosphorylated IRS1, as well as to play a role in regulating phosphatase catalytic activity (Kazlauskas *et al.*, 1993; Kuhne *et al.*, 1993; Lechleider *et al.*, 1993a,b; Case *et al.*, 1994; Li *et al.*, 1994; Sugimoto *et al.*, 1994; Kharitonenkov *et al.*, 1995). Since SH2 domain-mediated protein–protein interactions have been shown to always involve a conserved and essential Arg residue (Marengere and Pawson, 1992; Pawson and Gish, 1992), we addressed the question of the SH2 domain's significance for PTP1D tyrosine

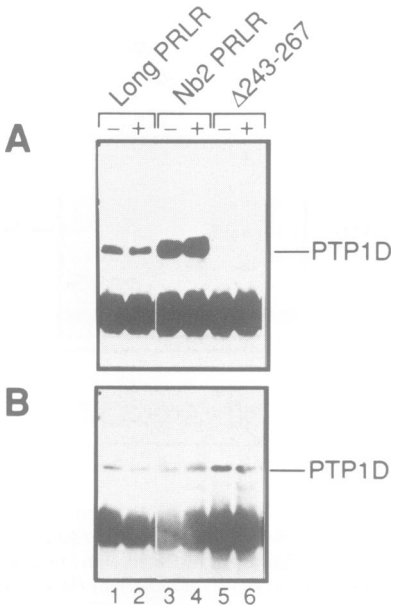

Fig. 4. The membrane-proximal region of PRLR is required for PRL-dependent PTP1D tyrosine phosphorylation. 293-LA cells were co-transfected with the human PTP1D expression plasmid and expression vectors containing cDNAs of either PRLR long form (Shirota *et al.*, 1990) (lanes 1 and 2), PRLR Nb2 form (Ali *et al.*, 1991) (lanes 3 and 4) or a mutant form (Δ243–268) (Lebrun *et al.*, 1995a). Cells were stimulated with oPRL (10^{-7} M) for 10 min (+) or left unstimulated (–). Processing of cells, immunoprecipitation and Western blotting were performed as described in the legend to Figure 3 and in Materials and methods. (**A**) Membrane immunodetected with monoclonal antibodies to phosphotyrosine (4G10). (**B**) Membrane detected with monoclonal anti-PTP1D antibodies.

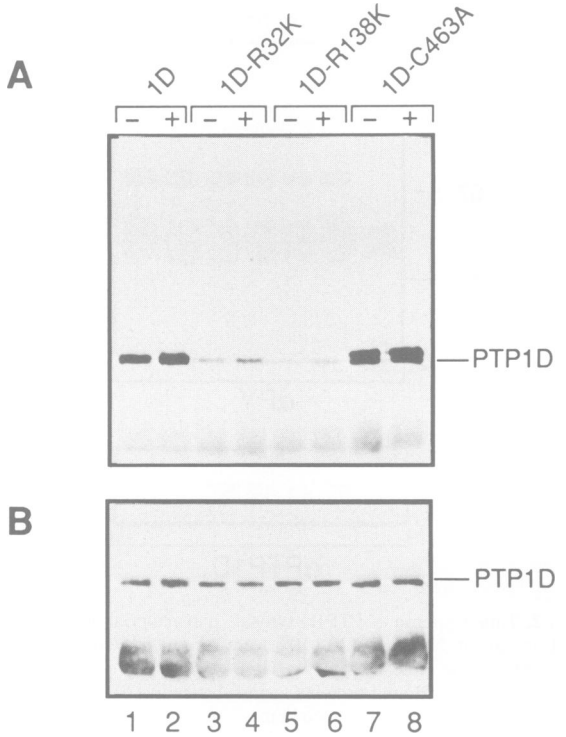

Fig. 5. Tyrosine phosphorylation of PTP1D is dependent on SH2–phosphotyrosine interactions as well as catalytic activity. 293-LA cells were transiently transfected with the PRLR long form expression plasmid and expression plasmids for either wild-type PTP1D (lanes 1 and 2), PTP1DR32K (lanes 3 and 4), PTP1DR138K (lanes 5 and 6) or PTP1DC463A (lanes 7 and 8). Following 24 h starvation cells were either left untreated (–) or treated (+) for 10 min with oPRL (10^{-7} M). PTP1D was immunoprecipitated from cell lysates. Proteins were separated by SDS–PAGE and transferred to nitrocellulose. (**A**) Membrane probed with a monoclonal antibody 4G10 to phosphotyrosine. (**B**) The same membrane stripped and reprobed with a monoclonal anti-PTP1D antibody.

phosphorylation and interaction with the PRLR–Jak2 complex by substituting this residue with Lys in either the N-terminal or C-terminal SH2 domain and testing the substrate activity of these mutants in the 293-LA co-transfection system. As shown in Figure 5A, both mutations resulted in a dramatic decrease in the ability of the PRLR–Jak2 complex to mediate PRL-dependent tyrosine phosphorylation of the phosphatase, emphasizing the importance of SH2/phosphotyrosine-mediated interactions for this process.

To investigate further the role of PTP1D we generated a mutant in which a conserved Cys residue (Cys463), known to be required for phosphatase catalytic function (Charbonneau and Tonks, 1992), was substituted by Ala. As depicted in Figure 5A, this mutation resulted in an increase in PTP1D phosphotyrosine content in co-transfections with the long form of PRLR in 293-LA cells, indicating that phosphatase function may negatively influence tyrosine phosphorylation by the PRLR–Jak2 complex.

Tyrosine phosphorylation of PRLR is not necessary for PTP1D phosphorylation

Since the SH2 domains of PTP1D are important for PRL-dependent tyrosine phosphorylation of PTP1D, we sought to ascertain whether phosphotyrosines present on PRLR were crucial for PTP1D recruitment and thereby tyrosine phosphorylation. Since we recently demonstrated that the short form of PRLR is able to associate with and activate Jak2 upon PRL stimulation without becoming tyrosine-phosphorylated itself (Lebrun *et al.*, 1995a), we examined the ability of this PRLR isotype to mediate ligand-induced PTP1D phosphorylation in the transient expression system described above. As shown in Figure 6, transient co-expression of PRLR short form and PTP1D in 293-LA cells resulted in tyrosine phosphorylation of the SH2 phosphatase. Thus phosphorylation of PRLR is not necessary for formation of the signaling complex and PRL-induced tyrosine phosphorylation of PTP1D.

PTP1D is a positive signal regulator in PRL-mediated β-casein gene transcription

Finally, we examined the functional significance of PTP1D in the PRL-triggered signal transduction pathway. Transcriptional regulation of the rat β-casein gene by this peptide hormone has been extensively studied (reviewed in Doppler, 1994) and we had previously developed a transient transfection assay system involving co-transfection of an expression plasmid encoding PRLR with a luciferase reporter gene under the control of the promoter region of the rat milk protein gene β-casein (Lebrun *et al.*, 1995a,b). In 293 cells co-transfected with expression vectors for the long form of PRLR and the fusion gene β(–2300/+490)–luciferase PRL stimulated luciferase activity 5.8 ± 2.1-fold. Co-overexpression of PTP1D along with PRLR long form in this system caused a 25%

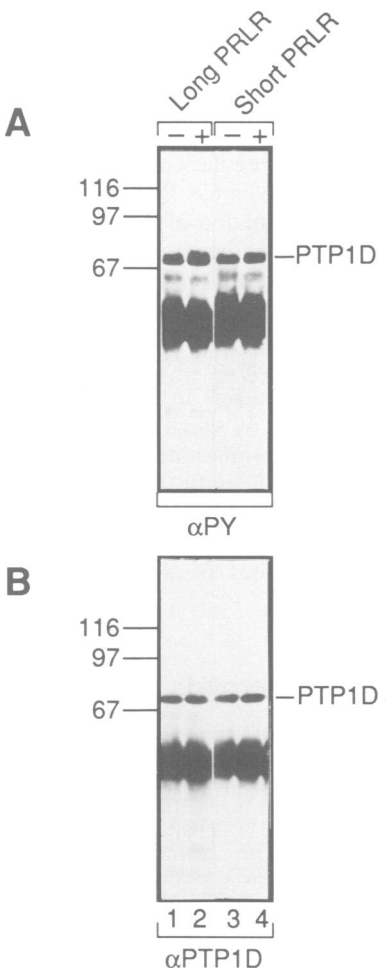

Fig. 6. The short form of PRLR mediates the signal to phosphorylate PTP1D. 293-LA cells were co-transfected with the human PTP1D expression plasmid and expression vectors containing cDNAs of either PRLR long form (lanes 1 and 2) or PRLR short form (Boutin *et al.*, 1988) (lanes 3 and 4). Following overnight starvation cells were stimulated with oPRL (10^{-7} M) for 10 min (+) or left unstimulated (–). Cell lysates were used for immunoprecipitation of PTP1D. Proteins were processed as described in Materials and methods for Western blotting. (**A**) Membrane probed with a monoclonal antibody 4G10 to phosphotyrosine. (**B**) The same membrane stripped and reprobed with a monoclonal anti-PTP1D antibody.

increase in PRL-induced activation of the β-casein gene promoter compared with control cells (Figure 7). In contrast, however, co-overexpression of a catalytically inactive mutant of PTP1D (PTP1DC463A) or mutants that abrogate SH2 interactions (PTP1DR32K and PTP1DR138K) with PRLR long form in this system reduced luciferase activity to ~50% of the control (Figure 7). This indicates that both mutations of PTP1D led to inhibition of PRL-induced lactogenic signaling, presumably by dominant negative competition with endogenous PTP1D. Moreover, this observation suggested that both binding of PTP1D SH2 domains to currently unidentified phosphotyrosines and an active phosphatase are necessary for transmission of the PRL signal.

Discussion

Following the initial interaction of PRL with its cell surface receptor the first molecular events that eventually

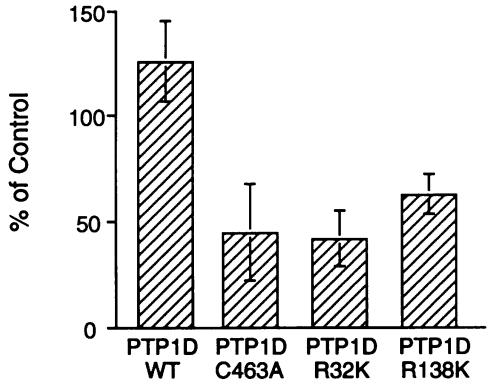

Fig. 7. Effects of expression of wild-type and mutant forms of PTP1D on PRL-mediated β-casein gene promoter activation. 293 cells were co-transfected with the expression plasmid encoding PRLR long form, the fusion gene carrying the promoter region of the β-casein gene linked to the coding region of the luciferase reporter gene (Lebrun *et al.*, 1995a,b), β-galactosidase expression vector pCH110 and different PTP1D expression plasmids. One day following transfection cells were incubated in starvation medium containing either dexamethasone (250 nM) alone as a control or stimulated with dexamethasone (250 nM) and oPRL (50 nM) for 24 h, before being lysed. Each value represents the stimulated luciferase activity in relative light units (RLU) (fold induction), normalized for β-galactosidase activity as percent maximal activity [the positive control is stimulated luciferase activity (fold induction) in the absence of overtransfection with PTP1D]. Results are the means ± SEM of three independent experiments.

lead to the overall response of the cell have recently been elucidated. PRLR, like other members of the cytokine receptor family lacking an intrinsic catalytic activity for generation and promotion of cellular signals, forms bimolecular signaling complexes with cytoplasmic tyrosine kinases of the Jak and Src families. Stimulation of the receptor–kinase complex by extracellular interaction with a ligand leads to activation and tyrosine phosphorylation of the associated kinase, the receptor and cellular proteins representing primary substrates or downstream signal transducers.

Here we investigated the role of SH2 domain-containing PTP1D in the PRL signal. In the Nb2 model system, which we used to study interaction of this phosphatase with PRLR, we find tyrosine phosphorylation of endogenous PTP1D in response to PRL stimulation. Moreover, results from co-immunoprecipitation studies in endogenous as well as in the 293 cell overexpression system provide evidence that PTP1D physically associates with preformed PRLR–Jak2 complex. While Jak2 has been identified before as the most proximal kinase activated following PRLR stimulation (Campbell *et al.*, 1994; Lebrun *et al.*, 1994; Rui *et al.*, 1994), the time course of phosphorylation of PTP1D indicates tight association of PTP1D with the PRLR–Jak2 complex and suggests Jak2 as the kinase involved in PTP1D phosphorylation. This does not exclude the possibility, however, of the presence of another tyrosine kinase activated by Jak2 that could in turn phosphorylate PTP1D.

Jak2 is a member of the Janus family of tyrosine kinases, which are characterized by the presence of two kinase-related domains and are implicated in the action of several cytokines (Ihle *et al.*, 1994). Our understanding of the mechanism of Jak2 activation and the effector molecules that act downstream of the receptor–kinase

complex is incomplete. It has been shown in different systems, however, that the kinase activity of Jak2 is highly dependent on prior ligand–receptor interaction (Silvennoinen *et al.*, 1993; Miura *et al.*, 1994) and that activation of Jak2 requires a proline-rich, membrane-proximal region which is conserved among the members of the cytokine receptor family (Da Silva *et al.*, 1994; Quelle *et al.*, 1994; Sotiropoulos *et al.*, 1994; Vander Kuur *et al.*, 1994; Lebrun *et al.*, 1995). The proposal that Jak2 is the kinase that phosphorylates PTP1D is further supported by the finding that in 293-LA cells stably overexpressing this enzyme no phosphorylation of the phosphatase was observed in cells expressing PTP1D alone or in cells co-expressing a mutant of PRLR lacking the Jak2 association domain. Thus our results establish PTP1D as a Jak2 substrate which is phosphorylated upon PRL stimulation. Tyrosine phosphorylation of PTP1D in response to other cytokines, such as IL3/GM-CSF (Welham *et al.*, 1994), IL6/CNTF/LIF (Boulton *et al.*, 1994) and erythropoietin (Tauchi *et al.*, 1995), may, because of the structural similarity of the receptors, also involve Jak2 or a closely related kinase. Similarly, tyrosine phosphorylation of SH2 transcription factors of the Stat family has been shown to be dependent on Jak2, although direct physical association has not been demonstrated (Larner *et al.*, 1993; Shuai *et al.*, 1993). In contrast, our experimental observations clearly support ligand-dependent formation of a trimeric complex between PRLR–Jak2 and PTP1D and the requirement of this event for phosphorylation of the phosphatase component.

This formation of a functional PRLR–Jak2–PTP1D signaling complex requires, in addition to the conserved proline-rich region in the receptor cytoplasmic region necessary for Jak2 association (Lebrun *et al.*, 1995a), both PTP1D SH2 domains and an intact phosphatase function. Interestingly, however, while mutations resulting in impairment of SH2 domain–phosphotyrosine interaction are inhibitory for PTP1D tyrosine phosphorylation, mutation-induced phosphatase inactivation in the PTP1DC463A mutant results in an enhanced phosphotyrosine content in cells co-expressing PRLR and Jak2, as well as in other receptor systems (Stein-Gerlach *et al.*, 1995). Whether this phenomenon reflects an autodephosphorylation activity of PTP1D or the involvement of another negative feedback regulation system is currently unclear. This observation suggests, however, that PTP1D tyrosine phosphorylation is necessary, but not sufficient, for further transmission of the PRL signal. Evidence confirming this conclusion is derived from our analysis of the effects caused by PTP1D mutations on one of the end points of the PRL signal, activation of the β-casein gene promoter. Here the inhibitory effects of SH2 domain R/K mutations observed in the phosphotyrosine analysis correlated with a similarly reduced transcriptional activity. In contrast, the phosphatase-inactivating C/A mutation of PTP1D, which caused hyperphosphorylation on tyrosine, resulted in significant suppression of β-casein promoter-regulated reporter gene expression.

Our observations significantly extend the previously postulated role of PTP1D in biological signal transduction, either as an adapter linking growth factor receptors to the Grb2–SOS complex, as shown in other systems (Bennett *et al.*, 1994; Li *et al.*, 1994; Tauchi *et al.*, 1994) or as an essential element for mitogenic signaling by insulin and other growth factors (Milarski and Saltiel, 1994; Noguchi *et al.*, 1994; Xiao *et al.*, 1994; Yamauchi *et al.*, 1995). Moreover, our results emphasize distinct functions of the two homologous SH2-containing phosphatases PTP1D and PTP1C in the regulation of signals generated by cytokine receptors. Whereas PTP1D is needed for transmission and/or potentiation of a signal, PTP1C has been previously shown to associate with the βc subunit of the IL-3 receptor and the EPO receptor and appears to cause receptor and/or Jak2 dephosphorylation and growth signal termination (Yi *et al.*, 1993; Klingmüller *et al.*, 1995).

Taken together, our experimental results establish PTP1D as an essential link in the PRL signaling cascade leading to activation of specific gene transcription. This signal control and transmission function of PTP1D requires an intact catalytic activity and two functional SH2 domains. Neither the substrate of the former, which may represent a negative regulator or localization element, nor the specific target sites of the two SH2 domains are currently known.

Materials and methods

Construction of expression plasmids

Construction of the expressions plasmids for the different PRLR forms and human PTP1D was performed as described previously (Vogel *et al.*, 1993; Lebrun *et al.*, 1995). PTP1D mutants containing Arg→Lys substitutions at position 32 (PTP1DR32K) and 138 (PTP1DR138K) or Cys→Ala (PTP1DC463A) were generated using PCR-based mutagenesis with the following oligonucleotide primers: 5′-TTACTAGGCTTTGCC-AAA-3′ for PTP1DR32K, 5′-CTGGCTCTCTTTTAGAAGAAA-3′ for PTP1DR138K and 5′-AGAATACTTAAGTTTGTAAATTCGTGCCC-3′ for PTP1DC463A.

Cell culture and transfection

The Nb2-11C prolactin-dependent cell line, developed and provided by Dr P.Gout (Vancouver, Canada), was grown in complete medium consisting of RPMI 1640 containing 10% fetal calf serum, 10% horse serum (Sigma catalog number H1895), 2 mM L-glutamine, 5 mM HEPES buffer, pH 7.4, penicillin/streptomycin (50 U/ml and 50 μg/ml respectively) and 50 μM 2 β-mercaptoethanol (βME) (Lebrun *et al.*, 1994). Following overnight incubation in starvation medium (identical to complete medium but without fetal calf serum and βME), cells were stimulated by oPRL (ovine prolactin, obtained from the National Health and Pituitary Program/NIDDK, Baltimore, MD) for the indicated times for individual experiments. Cells were quickly pelleted in an Eppendorf centrifuge and prepared for cross-linking with DSP (thiol cleavable cross linker, Pierce Chemical Co.) as described previously (Lebrun *et al.*, 1994).

Human 293-LA cells were grown in Dulbecco's modified Eagle's medium (DMEM) nut F12 with 10% fetal calf serum. Several hours before transfection cells were plated in a rich medium (2/3 DMEM nut F12, 1/3 DMEM 4.5 g/l glucose, 10% fetal calf serum). Exponentially growing cells were transiently transfected using the calcium phosphate method (Lebrun *et al.*, 1995a,b) with specific expression vectors as indicated in the figure legends. After 24 h serum starvation cells were stimulated for 10 min with oPRL (10^{-7} M) and collected for protein preparation.

Purification of PTP1D immune complexes

Cell pellets were solubilized in lysis buffer (50 mM HEPES, pH 7.5, 5 mM EDTA, 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 μM sodium pervanadate, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, 2 μg/ml leupeptin). Insoluble material was removed by 15 min centrifugation at 15 000 *g* at 4°C and the supernatants incubated with anti-PTP1D polyclonal antibody + 10 μl protein A–Sepharose beads (Pharmacia) for 3 h at 4°C. Samples were washed three times in 1 ml lysis buffer and eluted in 20 μl sample buffer. Samples were then boiled for 5 min before loading on SDS–polyacrylamide gels.

Western blot analysis

Proteins were separated on either 7.5 or 10% SDS–polyacrylamide gels and electrophoretically transferred onto nitrocellulose filters. For immunoblot analysis membranes were preincubated with 1% bovine serum albumin solution in TBST (20 mM Tris, pH 7.5, 150 mM NaCl, 0.02% Tween 20) and probed with either anti-phosphotyrosine antibody (0.1 μg/ml; 4G10, Upstate Biotechnology), an anti-PTP1D polyclonal antibody (Vogel *et al.*, 1993), an anti-PTP1D monoclonal antibody (1:1000 dilution; Transduction Laboratory) or anti-Jak2 antibody (dilution 1:4000; UBI) overnight. Membranes were then incubated with goat anti-mouse antibody or goat anti-rabbit antibody coupled to peroxidase for 1 h at room temperature before being washed four times for 30 min in TBST. The signal was then revealed by chemiluminescence (ECL kit, Amersham) according to the manufacturer's instructions.

Functional analysis

The 293 cell maintenance and transfection methods were performed as described for 293-LA cells. Cells were plated in 6-well plates (0.5×10^6 cells/well) in rich medium before being co-transfected with 0.1 μg expression plasmid encoding PRLR long form, 0.2 μg fusion gene carrying the promoter region of the β-casein gene linked to the coding region of the luciferase reporter gene, 0.5 μg pCH110 (β-galactosidase expression vector; Pharmacia) and 0.05 μg different PTP1D expression plasmids. One day after transfection cells were incubated in starvation medium containing either dexamethasone (250 nM) alone as a control or stimulated with dexamethasone (250 nM) and oPRL (50 nM) for 24 h, before being lysed.

Acknowledgements

We thank James Ihle for supplying the cDNA encoding the tyrosine kinase Jak2, Jeffrey Rosen for providing the β-casein promoter, Helen Buteau for preparing the fusion gene with the luciferase promoter, Esther Zwick for providing PTP1D mutants and Jeanne Arch for expert preparation of the manuscript. We are also grateful to the National Hormone and Pituitary Program for providing ovine PRL. S.A. is the recipient of a post-doctoral fellowship from the Fonds de Recherches en Santé du Québec. This work was supported by a grant from SUGEN Inc.

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Received on May 11, 1995; revised on September 26, 1995