The protein tyrosine phosphatase-BL, modulates pancreatic β-cell proliferation by interaction with the Wnt signalling pathway

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Abstract

In pancreatic β-cells, increased expression of the MODY5 gene product, HNF1β, leads to enhanced rates of apoptosis and altered regulation of the cell cycle, suggesting that control of HNF1β expression may be important for the control of β-cell proliferation and viability. It is unclear how these effects of HNF1β are mediated, but previously we have identified a protein tyrosine phosphatase, (PTP)-BL, as an HNF1β-regulated protein in β-cells and have now studied the role of this protein in INS-1 β-cells. Stably transfected cells were generated, which express either wild-type (WT) or a phosphatase-deficient mutant (PTP-BL-CS) of PTP-BL conditionally under the control of a tetracycline-regulated promoter. Enhanced expression of WT PTP-BL inhibited INS-1 cell growth dose dependently, but this effect was not observed when PTP-BL-CS was expressed. Neither construct altered the rate of apoptosis. PTP-BL has been reported to interact with components of the Wnt signalling pathway, and we observed that addition of exogenous Wnt3a resulted in an increase in cell proliferation and a rise in β-catenin levels, consistent with the operation of this pathway in INS-1 cells. Up-regulation of WT PTP-BL antagonised these responses but PTP-BL-CS failed to inhibit Wnt3a-induced proliferation. The rise in β-catenin caused by Wnt3a was also suppressed by over-expression of HNF1β, suggesting that HNF1β may interact with the Wnt signalling pathway via an increase in PTP-BL levels. We conclude that PTP-BL plays an important role in the regulation of cell cycle progression in pancreatic β-cells, and that it interacts functionally with components of the Wnt signalling pathway.

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Introduction

Maturity onset diabetes of the young (MODY) results from mutations arising in either glucokinase (MODY2) or any of several transcription factors involved in the regulation of the functional activity of the cells (Fajans et al. 2001, Stride & Hattersley 2002). Thus, identification of the proteins that are mutated in patients with MODY can provide important insights into the functional control of β-cell integrity. In this context, we have recently demonstrated that increased expression of the MODY5 gene product, HNF1β, leads to enhanced rates of apoptosis, altered regulation of the cell cycle and inhibition of stimulated insulin secretion in pancreatic β-cells, suggesting that control of HNF1β expression may be important for the regulation of β-cell viability and function (Welters et al. 2006).

HNF1β is a developmentally regulated protein whose levels of expression change dramatically during pancreatic embryogenesis. Embryonic β-cells express HNF1β in relatively large amounts, whereas it is expressed at very much lower levels in mature β-cells (Maestro et al. 2003). This may reflect the differentiation status of the β-cell and implies that tight regulation of HNF1β levels could be one component of the mechanism by which β-cell proliferation and apoptosis are controlled in maturity. Since HNF1β is a transcription factor, it is likely that the altered growth and viability caused by increased expression of this molecule result from the changes in the expression of one or more key target genes. Microarray analysis has revealed that a large number of genes are sensitive to HNF1β (Thomas et al. 2004), and we have begun to analyse these to identify previously unrecognised molecules that may be important for the control of β-cell viability.

One β-cell gene whose expression is significantly up-regulated in response to HNF1β encodes a protein tyrosine phosphatase, PTP-BL, which has not been previously studied in these cells. PTP-BL (ptpn13) is the rodent homologue of human PTP-bas (also called FAP-1, PTPL1 or hPTP1e (Maekawa et al. 1994, Saras et al. 1994)) and has been implicated in the control of survival and proliferation in other cell types (Bompard et al. 2002), suggesting that it may fulfil similar roles in the β-cell.

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PTP-BL is a non-receptor protein tyrosine phosphatase that is expressed during development, but is also present in a range of adult tissues, including pancreas, kidney, muscle, liver, heart, brain, and lung (Saras et al. 1994, Lee et al. 1999). It is a large modular protein (MW ~ 270 kDa) with multiple functional domains, including one KIND, one FERM and five PDZ domains in addition to the catalytic tyrosine phosphatase domain (reviewed in Erdmann 2003). The PDZ domains are involved in protein–protein interaction and can bind various effector proteins, including the tumour suppressor protein, adenomatosis polyposis coli (APC; Erdmann et al. 2000), and the death receptor, Fas, as well as a number of other signalling proteins (Saras et al. 1997). The FERM domain of PTP-BL may be involved in regulating the intracellular distribution of the enzyme since it can bind to PtdIns(4,5)P2 (Cuppen et al. 1999, Bompard et al. 2003), which could be important for targeting the phosphatase to relevant substrates located at the plasma membrane. However, an important substrate of the phosphatase domain is the soluble intracellular protein β-catenin (Erdmann et al. 2000) that plays a role in the regulation of both cell adhesion and gene transcription (Harris & Peifer 2005, Brembeck et al. 2006).

In view of the evidence that expression of PTP-BL is increased in β-cells having elevated levels of HNF1β, and that this correlates with altered proliferation under these conditions (Welters et al. 2006), the present study has investigated the role of PTP-BL in mediating these changes. To achieve this, a β-cell line conditionally expressing PTP-BL under the control of a tetracycline-regulated promoter was generated and the effects of altered expression of PTP-BL studied.

Materials and Methods

Cell culture

The rat β-cell line INS-1 was cultured in RPMI-1640 medium containing 11 mM glucose, 10% fetal bovine serum, 2 mM l-glutamine, 50 μM β-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C. INS-1 Flp-In T-REx parental cell clone #1-1.2 (Thomas et al. 2004) in combination with the pOG44 plasmid, encoding Flp recombinase, using TransFAST reagent, as described previously (Thomas et al. 2004). Cells in which successful recombination had occurred were selected by growth in 150 μg/ml hygromycin.

Measurement of cell proliferation

Cell proliferation was measured with the CellTiter 96 aqueous one solution cell proliferation assay (Promega). Cells were seeded onto 96-well plates at 1×10³ cells/well, 24 h prior to tetracycline induction. At the end of the induction period (which varied according to the experiment), 100 μl fresh media plus 20 μl cell proliferation assay solution was added per well and incubated at 37 °C for 2 h before measurement of the absorbance at 490 nm using a Tecan GENios spectrophotometer.

Estimation of cell viability with trypan blue

For the determination of cell viability, vital dye staining was used. Experiments were carried out on 6-well plates with 1×10⁵ cells/well seeded 24 h before induction of PTP-BL expression. Floating and attached cells were collected from each well and stained with trypan blue. The number of viable and dead cells was counted using a haemocytometer.

Caspase assay

CaspACE FITC-VAD-FMK In Situ Marker (Promega), a fluorosorothiocyanate conjugate of the cell-permeable caspase inhibitor VAD-FMK, can be localised by fluorescence detection and acts as an in situ marker for cells undergoing apoptosis. Treated cells were labelled with 10 mM CaspACE, according to the manufacturer’s instructions, and viewed by fluorescence microscopy. The number of green fluorescent cells was counted in a field of about 100 cells and the percentage of apoptosis calculated.

Flow cytometric (FACS) analysis

Cells were harvested and re-suspended in 200 μl PBS before the addition of 2 ml ice-cold ethanol:PBS (70%;30%; vol:vol) and incubated on ice for 30 min. The cells were then collected and the pellet re-suspended in 1 ml propidium iodide (0.5 mg/ml) containing 2-5 μg RNase (DNase free) and incubated for 30 min at 37 °C. The distribution of cellular DNA was subsequently analysed on a FACS can flow cytometer (Becton Dickinson, Oxford, Oxfordshire, UK).
Insulin secretion assays

Cells were seeded onto 24-well plates at 1 × 10⁵ cells per well, 24 h before the addition of tetracycline. At the end of the induction period, the cells were pre-incubated for 90 min in 500 μl incubation buffer (Gey & Gey 1936) containing 6 mM glucose and 0.1% BSA. They were then stimulated acutely with the test reagents and the incubation medium was sampled after 1 h for the measurement of insulin by RIA.

Western blotting

To extract whole INS-1 cell protein, cells were washed with ice-cold PBS before the addition of 0.2 ml lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA and 1% Triton X, with 10 μl/ml protease inhibitor cocktail (Sigma) added just before use) per 25 cm² flask, for 10 min, on ice. The flasks were then scraped, the contents transferred to a microfuge tube (on ice) and vortexed (4 × 15 s), with 5 s on ice between each vortexing. The protein extract was then centrifuged at 1000 × g for 10 min at 4 °C and the supernatant stored at −80 °C.

Equal amounts of denatured protein samples were run on a pre-cast bis–Tris–HCl-buffered 12% polyacrylamide gel (Invitrogen) at 200 V for 1 h in MOPS SDS running buffer (50 mM 3-(N-morpholino)-propanesulphonic acid, 50 mM Tris base, 3.5 mM SDS, 1 mM EDTA).

A prestained marker set (Amersham) was included to allow the sizes of relevant bands to be determined. Proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore) using a ‘wet’ transfer tank (Bio-Rad Trans blot cell) for 4 h at 250 mA. The membrane was blocked overnight at 4 °C in Tris-buffered saline containing 0.05% Tween (TTBS) and 5% low fat dried milk. Primary antibodies (PTP-BL; Prof. K Erdmann, p-T yr; Santa Cruz (sc-18182), β-catenin; R&D systems, phospho-β-catenin (Ser45); Cell signalling (9564), active β-catenin; Upstate (05-665)) were diluted 1:2000 in TTBS containing 1% milk and incubated with the membrane for 4 h at room temperature. The appropriate (either anti-rabbit (Sigma, A3687) or anti-mouse (Sigma A3562)) IgG–alkaline phosphatase-conjugated secondary antibody was diluted 1:30 000 in TTBS containing 1% milk, added to the membrane and incubated for 1 h at room temperature. Immunoreactive bands were visualised using CDP-Star (Sigma) and exposure to X-ray film.

Immunoprecipitation

Protein A acrylic beads (Sigma) were used for immunoprecipitation. The beads were re-hydrated and blocked for 1 h in 10% goat serum. Cell protein lysates (100 μl) were preclered by the addition of 50 μl blocked protein A beads for 1 h at 4 °C. Ten micrograms of PTP-BL antibody were then added to the precleared lysates and incubated at 4 °C overnight before the addition of a further 50 μl protein A beads for 1 h at 4 °C. Following incubation, the beads were washed three times with 500 μl lysis buffer and re-suspended in 40 μl running buffer (containing 25% 4 × (LDS) lithium dodecyl sulphate sample buffer (Invitrogen), 10% β-mercaptoethanol and 65% lysis buffer) and boiled for 10 min. The supernatants were electrophoresed on a 5% Tris–HCl gel and western blotting carried out as described above.

p-Tyr ELISA

Levels of protein tyrosine phosphorylation were measured using a cell-based phosphotyrosine ELISA kit (RayBiotech, Norcross, GA, USA), according to the manufacturer’s instructions. Briefly, cells were seeded at 4 × 10⁶ cells per well, 24 h before the addition of tetracycline. Following induction (24 h), the cells were treated with test reagents for 15–30 min, fixed and then blocked in the plates. Anti-phosphotyrosine-HRP was added and incubated for 1 h at room temperature. After washing to remove excess antibody, tetramethylbenzidine was added for 30 min followed by sulphuric acid to stop the reaction. The absorbance was measured at 450 nm in a Tecan GENios spectrophotometer.

Wnt signalling array

RNA was extracted from INS-1 cells using the Qiagen R.Neasy kit and cDNA was then synthesised from 1 μg RNA (First Strand cDNA Synthesis kit from SuperArray), according to the manufacturer’s instructions. The cDNA was used as a template for a PCR array employing a SYBR Green PCR master mix (Rat Wnt Signalling Pathway RT2 Profiler PCR Array; SuperArray cat no. APRN-043A). The PCR was run on a Bio-Rad iCycler, with 1 cycle of 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Cycle threshold (Ct) values were calculated for all the genes present on the array.

Statistical analysis

All individual experiments were performed in at least duplicate and were repeated on a minimum of three separate occasions. The results were analysed by ANOVA and were considered significant when P < 0.05.

Results

Effects of PTP-BL expression on INS-1 cell growth and viability

Previous microarray analysis had revealed that the transcript encoding PTP-BL becomes up-regulated in INS-1 cells induced to over-express wild-type HNF1β (Thomas et al. 2004). This was confirmed at the protein level by western blotting (Welters et al. 2006); thus PTP-BL has been established as an HNF1β-sensitive gene product in INS-1 cells.

In order to study the function of PTP-BL further, we exploited the potential of INS-1 Flp-In T-REx cells to stably...
incorporate WT PTP-BL cDNA under the control of a tetracycline-regulated promoter. Using this system, the levels of PTP-BL in INS-1 cells were increased substantially and in a dose-dependent manner as the tetracycline concentration was raised, consistent with the expected activity of the inducible promoter (Fig. 1). To confirm the functionality of the phosphatase activity of the expressed enzyme, the total extent of cellular protein tyrosine phosphorylation was measured by ELISA (Fig. 2). Incubation with IGF-I was selected as a means to promote an increase in protein tyrosine phosphorylation and, as expected, this agent caused a large rise in cellular tyrosine phosphorylation in INS-1 cells (Fig. 2). This increase was prevented in cells over-expressing PTP-BL, confirming that the phosphatase domain of the transfected PTP-BL is functionally active in pancreatic β-cells.

Since one of the consequences of an increase in HNF1β expression in INS-1 cells is a dramatic reduction in cell proliferation and an increase in apoptosis, the changes in cell growth and viability resulting from over-expression of PTP-BL were investigated to establish whether the increased expression of PTP-BL might underlie the responses to HNF1β. INS-1 cells induced to express PTP-BL displayed a marked attenuation in cell growth over 72 h as measured by MTS reduction (Fig. 3A). This response was dose dependent in that the highest level of induction resulted in the lowest proliferation rate but even a modest increase in PTP-BL (as seen when induced with 20 ng/ml tetracycline (Fig. 1)) caused a substantial inhibition of growth (Fig. 3A). These results corresponded with a decline in the total cell number after 72-h treatment (20 ng/ml tet; 76.7 ± 0.7% versus control (100%); 1 μg/ml tet; 54.2 ± 0.4% versus control; P<0.001).

In an attempt to separate the potential structural scaffolding properties of PTP-BL from its phosphatase activity, a further cell line conditionally expressing an enzymatically deficient mutant having a single amino acid substitution in the phosphatase domain of PTP-BL (PTP-BL-CS) was employed (Fig. 3B). In contrast to WT PTP-BL, high levels of PTP-BL-CS expression (induced with 1 μg/ml tetracycline) did not cause any inhibition of cell growth, even after 72 h. Indeed, induction of PTP-BL-CS caused a small increase in INS-1 cell growth. Treatment of control cells (transfected

Figure 1  Conditional expression of PTP-BL protein in INS-1 cells. INS-1 cells conditionally expressing PTP-BL were treated with 0, 5, 20 and 1000 ng/ml tetracycline (tet) for 24 h. Whole cell protein was extracted and western blotted with PTP-BL antibody. β-actin staining was used as a protein loading control.

Figure 2  Increased PTP-BL expression inhibits IGF-I induced increases in protein tyrosine phosphorylation. INS-1 cells conditionally expressing PTP-BL were treated with 0, 20 and 1000 ng/ml tetracycline (tet) for 24 h. The cells were then acutely stimulated with 10 nM IGF-I for 15 min and the levels of protein tyrosine phosphorylation measured by ELISA. Results shown as mean ± S.E.M. (n=6). *P<0.001 compared with uninduced control. #P<0.001 compared with uninduced cells treated with IGF-I.

Figure 3  Increased PTP-BL expression inhibits INS-1 cell growth. (A) INS-1 cells conditionally expressing PTP-BL were treated with 0 (○), 20 (●) and 1000 (□) ng/ml tetracycline (tet) and cell growth measured at the given time points. (B) INS-1 cells expressing wild-type PTP-BL (PTP-BL-WT) or the phosphatase-inactive mutant (PTP-BL-CS) were treated in the absence (uninduced) or presence (induced) of 1000 ng/ml tetracycline for 72 h. Cell growth was measured using a cell proliferation assay (Promega). Results shown as mean ± S.E.M. (n=6). *P<0.001 decreased compared with uninduced cells. #P<0.001 increase compared with uninduced cells.
with empty vector) with a high concentration of tetracycline (tet) (1 μg/ml) failed to affect their rate of proliferation over the time period of the studies (not presented). Thus, these results confirm that increased expression of PTP-BL causes a marked reduction in INS-1 cell growth, which is dependent on the enzyme activity of the phosphatase domain.

By contrast, and unlike the situation when HNF1β was up–regulated in INS-1 cells (Welters et al. 2006), there was no net loss of viability in cells over-expressing PTP-BL (cell death after 72 h of PTP-BL induction: control, 5.27 ± 0.4%; 1 μg/ml tet, 6.6 ± 0.9; not significant). This observation was confirmed by the measurement of caspase activity, which showed no significant increase in the level of apoptosis in cells over-expressing PTP-BL (% caspase positive cells after 72 h of PTP-BL induction: control, 1.09 ± 0.26%; 1 μg/ml tet, 1.09 ± 0.26%). This suggests that the primary effect of PTP-BL was to cause a reduction in growth rate rather than an increase in cell death.

To analyse further the effects of PTP-BL on cell proliferation, cell cycle analysis was performed by flow cytometry after staining with propidium iodide (Fig. 4). In uninduced cells, 50% of the population were found to be in the G1 phase of the cell cycle. Induction of PTP-BL increased the proportion of cells in the G1 phase significantly with a corresponding decrease in the number of cells in the G2/M phase. These effects were dose dependent with significant changes seen within 24 h following induction with 20 ng/ml tetracycline, and still larger changes observed after the addition of 1 μg/ml tetracycline.

Effects of PTP-BL on Wnt signalling in INS-1 cells

In addition to displaying tyrosine phosphatase activity, PTP-BL has also been demonstrated to interact with cellular proteins by virtue of its various protein binding cassettes (Saras et al. 1994, Erdmann et al. 2000, Erdmann 2003). Indeed, these two mechanisms are not exclusive since some of its potential binding partners are likely to be associated with tyrosine kinase substrates that are involved in the regulation of cell growth. Among these is the tumour suppressor protein, APC (Erdmann et al. 2000), that can form a complex with the transcription factor, β-catenin. This is significant since β-catenin has been reported to serve as a substrate for the tyrosine phosphatase activity of PTP-BL (Erdmann et al. 2000) and, together with APC, it forms an integral component of the canonical Wnt signalling pathway involved in growth regulation. Wnt signalling has not been studied in detail in mature β-cells, but very recent data imply that Wnt ligands can promote the proliferation of mouse β-cells (Rulifson et al. 2007).

To monitor the expression of components of the Wnt signalling pathway in INS-1 cells, we used a multiplex PCR array system (SuperArray, Rat Wnt Signalling Pathway PCR Array) (Table 1). Analysis of the data revealed that a range of components of the Wnt pathway are expressed at the mRNA level in INS-1 cells, including several frizzled receptors, a number of Wnt ligands and various members of the Wnt-regulated ‘destruction complex’, notably β-catenin, APC, axin and GSK3. In addition, the transcriptional partners of β-catenin, TCF and Lef are also present. These results suggest that all of the key components required for functional Wnt signalling are present in INS-1 cells.

On this basis, it is possible that one mechanism, by which the effects of over-expression of PTP-BL in β-cells might be manifested, is through the alteration of components of the Wnt signalling pathway. In order to test this hypothesis, we initially sought firm evidence that PTP-BL can bind to APC in β-cells. Accordingly, protein lysates from INS-1 cells were treated with a specific anti-PTP-BL antibody and the resulting immunoprecipitates were recovered and probed for the presence of APC (Fig. 5). Uninduced cells displayed a specific band corresponding to APC after immunoprecipitation with PTP-BL. This band was absent from cell lysates treated in the absence of the precipitating anti-PTP-BL antibody. Induction of PTP-BL protein caused an increase in the amount of precipitated APC protein, confirming that PTP-BL protein binds to the APC protein in INS-1 cells.

We then proceeded to investigate whether these components were altered in a manner consistent with the operation of the Wnt signalling pathway in INS-1 cells. Two initial approaches were used. First, INS-1 cells were treated with an inhibitor of GSK3β, which is expected to activate the Wnt signalling pathway by stabilising β-catenin and preventing its proteasomal degradation (Coghlan et al. 2000). As predicted, treatment of INS-1 cells with a potent and selective GSK3β inhibitor (SB216763) led to increased cell growth during a 72-h period (Fig. 5B). This result is consistent with a possible role for components of the Wnt signalling pathway in regulating INS-1 cell proliferation but, since GSK3β is also involved in other signalling pathways, a
Table 1 RT-PCR array of Wnt signalling pathway components in INS-1 cells. RNA was extracted from INS-1 cells (in duplicate) and the SuperArray RT² Profiler PCR array was used to measure mRNA expression of components of the Wnt signalling pathway, by real-time RT-PCR. Values are displayed as the mean Ct (threshold cycle) values (n=2). Ct values greater than 32 were considered below the threshold detection level. Ct values of housekeeping genes are displayed for comparison.

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APC, adenomatosis polyposis coli; Dkk, dickkopf homolog; Frzb, frizzled-related protein; Fzb, frizzled homologue; GSK, glycogen synthase kinase; Lrp, low-density lipoprotein receptor-related protein; Tcf, transcription factor; Ripl1, ribosomal protein large P1; Hprt, hypoxanthine guanine phosphoribosyl transferase; Rpl13a, ribosomal protein L13A; Ldha, lactate dehydrogenase; Actb, β-actin.

more direct approach was also employed. Addition of purified Wnt3a to INS-1 cells over a 72-h period increased their growth and the extent of this increase was very similar to that found with the GSK3β inhibitor (Fig. 5B). Most significantly, treatment of the cells with 20 ng/ml tetracycline (to induced modest over-expression of WT PTP-BL) in combination with either the GSK3β inhibitor or the exogenous Wnt3a abolished the ability of these compounds to promote cell growth (Fig. 5B). By contrast, increased expression of the phosphatase-deficient mutant, PTP-BL-CS, did not attenuate the increase in cell growth promoted by the GSK3β inhibitor or Wnt3a treatment (Fig. 5C).

In order to confirm that these effects were mediated by changes at the level of the β-catenin/APC complex, the effect of Wnt3a on β-catenin protein levels was also studied (Fig. 6A). In untreated cells, β-catenin was readily detected by western blotting and its levels were unchanged following up-regulation of PTP-BL expression with tetracycline. By contrast, exposure of cells to Wnt3a resulted in a marked increase in β-catenin levels, consistent with reduced proteasomal degradation under these conditions. Importantly, the increase in β-catenin seen in response to Wnt3a was prevented following induction of PTP-BL (Fig. 6A).

Levels of various phosphorylated forms of β-catenin were also measured to assess the influence of PTP-BL. Among these, the phosphorylation of Ser45 is of particular significance since this represents a priming site whose phosphorylation is required for the subsequent GSK3-mediated phosphorylation of Thr41, Ser37 and Ser33 (thereby leading to β-catenin degradation). However, we observed that Ser45 phosphorylated β-catenin was relatively low in abundance and was unchanged by induction of PTP-BL expression (Fig. 6B) or by the addition of Wnt3a (not shown). By contrast, the levels of ‘activate β-catenin’ (measured with an antibody that recognises specifically a form of the molecule that lacks phosphorylation on Ser37 and Thr41, the target sites for GSK3 phosphorylation) were more abundant in control cells but were decreased upon PTP-BL expression (Fig. 6B). This is consistent with the concept that PTP-BL may regulate components of the Wnt/β-catenin signalling pathway as a means to control the growth of pancreatic β-cells.

Finally, to confirm more directly that a rise in PTP-BL might underlie the ability of HNF1β to mediate its growth regulatory effects in β-cells, and to consider whether this may relate to alterations in the Wnt signalling pathway, the expression of β-catenin was also studied in cells exposed to Wnt3a under control conditions or after induction of HNF1β (Fig. 6C). As observed for cells over-expressing PTP-BL directly (Fig. 6A), up-regulation of HNF1β also antagonised the increase in β-catenin seen upon incubation of INS-1 cells with Wnt3a (Fig. 6C).

Effects of PTP-BL on β-cell secretory function

In a final series of experiments, the insulin secretory responses of INS-1 cells over-expressing PTP-BL were studied (Fig. 7). These experiments revealed that increased expression of
PTP-BL had no significant effect on the rise in insulin secretion elicited by incubation of cells with methyl succinate (an anaplerotic stimulus), α-ketoisocaproate or depolarisation with KCl. Thus, unlike an increase in HNF1β, which impairs insulin secretion (Welters et al. 2006), PTP-BL appears to regulate β-cell growth without directly altering the secretory function of the cells.

Figure 5 Interaction of PTP-BL with the Wnt signalling pathway. (A) INS-1 cells conditionally expressing PTP-BL were treated in the absence (lanes 1 and 3) or presence (lane 2) of 1 μg/ml tetracycline for 24 h. Whole cell protein was extracted and immunoprecipitated using an antibody specific to PTP-BL (lanes 1 and 2). The control (lane 3) contained no precipitating antibody. Precipitated proteins were run on a 5% Tris–HCl gel and western blotted for APC protein. INS-1 cells conditionally expressing PTP-BL-WT (B) or PTP-BL-CS (C) were treated in the absence (uninduced) or presence (induced) of 20 ng/ml tetracycline for 24 h in combination with 5 μM SB216763 (GSK3 inhibitor) or 10 ng/ml Wnt3a protein. Cell proliferation was measured after this time. Results shown as mean ± S.E.M. (n = 8). *P<0.001 compared with control, #P<0.001 compared with uninduced cells in the presence of the relevant agonist.

Figure 6 Induction of PTP-BL or HNF1β inhibits Wnt-stimulated increases in β-catenin protein levels. INS-1 cells conditionally expressing PTP-BL WT (A) or HNF1β WT (C) were treated with 1 μg/ml tetracycline (tet) in the presence or absence of 10 ng/ml purified recombinant mouse Wnt3a for 24 h. Whole cell protein was extracted and western blotted for β-catenin. β-actin staining was used as a protein loading control. Conditions were performed in duplicate and are representative of three independent experiments. Densitometry measurements were carried out and shown as a ratio of β-catenin to β-actin expression. *P<0.01 compared with control, #P<0.01 compared with cells treated with Wnt alone. INS-1 cells conditionally expressing PTP-BL WT (B) were treated with control media (control) or with 1 μg/ml tetracycline (tet) for 24 h. Whole cell protein was extracted and western blotted for active β-catenin or p-Ser45 β-catenin. β-actin staining was used as a protein loading control. Conditions were performed in duplicate and are representative of three separate experiments.
Discussion

Previous studies suggest that ptpn13 is a HNF1β-sensitive gene in β-cells (Thomas et al. 2004), and we have confirmed this by demonstrating a marked rise in PTP-BL protein levels following induction of HNF1β-expression in the INS-1 Flp-In T-REx cell (Welters et al. 2006). PTP-BL has not previously been studied in β-cells, but we have also confirmed that it is expressed in both normal rat and human islets by western blotting (H J Welters & N G Morgan, unpublished observations).

Since PTP-BL is regulated in an HNF1β-sensitive manner in β-cells, we considered whether some of the phenotypic changes associated with altered expression of HNF1β could be mediated by an increase in PTP-BL. Up-regulation of HNF1β results in impaired insulin secretion, increased apoptosis and a reduction in the rate of cell proliferation in INS-1 cells (Welters et al. 2006) but analysis of these responses revealed that they are differentially sensitive to PTP-BL. In particular, enhanced expression of PTP-BL did not lead to any attenuation of the insulin secretory response to either nutrient or depolalising stimuli. In addition, there was no increase in apoptosis in cells expressing elevated levels of PTP-BL. By contrast, the attenuation of β-cell proliferation seen upon induction of HNF1β (Welters et al. 2006) was mirrored by the expression of PTP-BL, consistent with the possibility that PTP-BL may play a role in the regulation of β-cell growth.

It is unclear whether a reduction in PTP-BL affects islet cell mass in vivo as in one study (Wansink et al. 2004), global deletion of the tyrosine phosphatase domain did not lead to any change in circulating insulin concentrations in mice. This could be taken to imply that total islet mass was not increased significantly, although possible changes in this parameter were not reported directly. It is though conceivable that a modest increase might have occurred without any overt alteration in circulating insulin if the β-cell glucose-sensing mechanism remained functionally intact.

The growth inhibitory effects of PTP-BL were only evident in cells expressing an enzyme with a functional tyrosine phosphatase domain and were lost when a phosphatase-deficient mutant of PTP-BL was expressed. This implies very strongly that PTP-BL exerts its influence by the dephosphorylation of tyrosine residues, potentially of proteins involved in growth stimulatory residues, rather than due to any structural or scaffolding properties.

One of the defining characteristics of PTP-BL is the possession of a range of different binding domains that allow it to recruit various protein partners, thereby altering the intracellular distribution of these molecules and bringing potential substrates into close proximity (Erdmann 2003, Wansink et al. 2004). In other cell types, the tumour suppressor protein, APC, has been shown to interact with the PDZ domains of PTP-BL (Erdmann et al. 2000), and this brings the phosphatase domain into close apposition with β-catenin (since β-catenin also binds to APC). As a result, PTP-BL is able to dephosphorylate β-catenin at relevant tyrosine residues (Erdmann et al. 2000), and this may have consequences for the functional activity of the protein.

We have shown by immunoprecipitation that APC can bind to PTP-BL in β-cells, which supports the possibility that PTP-BL may play a role in regulating β-catenin levels and thereby cell growth. Consistent with this, it was observed that exposure of INS-1 cells to the GSK3β inhibitor, SB216763, caused an increase in cell proliferation (as expected if β-catenin levels are increased under these conditions). Most importantly, this response was abrogated upon mild elevation of PTP-BL.

In order to establish the significance of these effects of PTP-BL more directly, we examined the response to a physiological agonist that controls cell growth via the APC/β-catenin system, Wnt3a. It has been reported previously that β-cells express receptors for Wnt ligands (Heller et al. 2003), and we have confirmed this (Table 1). Recent evidence has indicated that the Wnt pathway is active in mature β-cells (Fujino et al. 2003, Rulifson et al. 2007, Schinner et al. 2008). Moreover, a transcription factor involved in the Wnt signalling pathway, TCF7L2, has been demonstrated to be required for β-cell survival in human islets (Shu et al. 2007). Moreover, a polymorphic variant of TCF7L2 has been strongly implicated in conferring increased susceptibility to type 2 diabetes (Grant et al. 2006), implying that Wnt signalling may be critical for β-cell maturation and functionality.

We have provided direct evidence that Wnt3a can signal via the APC/β-catenin system in β-cells as exposure of these cells to Wnt3a caused an increase in proliferation, and this was associated with a rise in β-catenin levels. The stimulation of β-cell growth by Wnt3a has also been reported very recently in isolated mouse islets (Rulifson et al. 2007, Schinner et al. 2008), suggesting that INS-1 cells behave in a similar manner.
to primary β-cells in this respect. In the present work, we observed that the proliferative response of INS-1 cells to exogenous Wnt3a was prevented by increased expression of PTP-BL. These data reveal that the Wnt signalling pathway is subject to regulation by PTP-BL in β-cells. In this context, it is of interest to note that a highly homologous protein tyrosine phosphatase, ‘FRIED’, has been identified in Xenopus where it regulates Wnt signalling (Itoh et al. 2005). In addition, PTP-BL was able to decrease cell growth in the absence of stimulation by Wnt3a. Analysis of β-catenin phosphorylation revealed that the ‘active’ form of the protein (which lacks phosphorylation on the target residues for GSK3 phosphorylation) was relatively abundant in untreated cells, in agreement with the recent report of Liu & Habener (2008) who showed that β-cells have a high basal level of activity in the Wnt signalling pathway. Importantly, we found that the levels of active β-catenin were decreased upon induction of PTP-BL expression, thereby providing strong support for the hypothesis that PTP-BL inhibits β-cell proliferation by antagonising the Wnt signalling pathway.

Since PTP-BL expression is increased in response to a rise in HNF1β, we considered it important to verify the prediction that up-regulation of HNF1β causes a decrease in β-catenin protein levels in INS-1 cells exposed to Wnt3a. Effects of HNF1β on β-catenin levels would not have been detected during earlier RNA microarray analysis of HNF1β-sensitive genes since it results from altered protein stability rather than from a direct change in gene transcription. In confirmation of the prediction that HNF1β can regulate β-catenin levels, western blotting revealed that the rise in β-catenin protein seen in INS-1 cells exposed to Wnt3a was attenuated upon induction of HNF1β. Thus, when considered together, the present data show that HNF1β regulates the Wnt signalling pathway in β-cells and they imply that this effect may be secondary to increased expression of PTP-BL.

In summary, the present results have provided evidence that components of the Wnt signalling pathway may play an important role in regulating the growth of mature β-cells and have shown that this pathway is subject to regulation by HNF1β. There has been recent in vivo evidence that supports a role for the modulation of Wnt signalling by HNF1β. For example, in two foetuses with mutations in HNF1β, levels of β-catenin were found to be increased in the kidney, but decreased in the exocrine and endocrine pancreas (Haumont et al. 2006). Our current study has implicated the multi-functional tyrosine phosphatase, PTP-BL, in mediating some of the effects of HNF1β and has revealed that PTP-BL may influence β-cell proliferation by regulating β-catenin levels. Since HNF1β expression is compromised in patients with MODY5, the results imply that changes in PTP-BL expression and thereby Wnt signalling could underlie some of the β-cell dysfunction seen in these patients.

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