Phosphorylation Marks IPF1/PDX1 Protein for Degradation by Glycogen Synthase Kinase 3-dependent Mechanisms*

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The transcription factor IPF1/PDX1 plays a crucial role in both pancreas development and maintenance of β-cell function. Targeted disruption of this transcription factor in β-cells leads to diabetes, whereas reduced expression levels affect insulin expression and secretion. Therefore, it is essential to determine molecular mechanisms underlying the regulation of this key transcription factor on mRNA levels and, most importantly, on protein levels. Here we show that a minor portion of IPF1/PDX1 is phosphorylated on serine 61 and/or serine 66 in pancreatic β-cells. This phosphorylated form of IPF1/PDX1 preferentially accumulates following pro- tease inhibition, an effect that is prevented by inhibition of glycogen synthase kinase 3 (GSK3) activity. Oxidative stress, which is associated with the diabetic state, (i) increases IPF1/PDX1 Ser61 and/or Ser66 phosphorylation and (ii) increases the degradation rate and decreases the half-life of IPF1/PDX1 protein. In addition, we provide evidence that GSK3 activity participates in oxidative stress-induced effects on β-cells. Thus, this current study uncovers a new mechanism that might contribute to diminished levels of IPF1/PDX1 protein and β-cell dysfunction during the progression of diabetes.

The homeodomain transcription factor IPF1/PDX1 (insulin promoter factor 1/pancreas duodenum homeobox 1) operates as a master of pancreatic development and also ensures differentiated β-cell function. Homozygous disruption of the Ipf1/Pdx1 gene results in pancreatic agenesis in both mice (1, 2) and humans (3). Moreover, targeted disruption of the Ipf1/Pdx1 gene selectively in β-cells leads to diabetes in mice (4), whereas partial reductions of IPF1/PDX1 expression levels in genetically modified mouse models affect insulin expression (4) and insulin secretion (5) and predispose islets to apoptosis (6). In humans, heterozygosity for an inactivating mutation in the Ipf1/Pdx1 gene is associated with development of (MODY) 4) maturity onset diabetes of the young (7), a monogenic form of type 2 diabetes that results from impaired β-cell function characterized by perturbed insulin secretion. Moreover, missense mutations in the Ipf1/Pdx1 gene, in conjunction with other mutations, appear to predispose to the adult onset form of type 2 diabetes (8–11). These results strongly support an essential role for IPF1/PDX1 in pancreas development and β-cell function that is conserved from mice to humans.

The participation of IPF1/PDX1 in the transcription of several genes essential for glucose sensing and insulin synthesis underlines its key role in differentiated β-cells. IPF1/PDX1 has been shown to bind and regulate the promoter activity of specific β-cell genes such as insulin (12), Glut2 (13), glucokinase (14), and islet amyloid polypeptide (15, 16). At the level of the insulin gene promoter, IPF1/PDX1 acts in concert with other transcription factors, such as beta2/Neuro D1 and the E2A family proteins, and coactivators including p300 and Bridge-1 (17–21). Thus, it is the accurate protein-protein interactions between transcription factors and coactivators that define the level of expression of the insulin gene.

Despite a clearly established role for IPF1/PDX1 in pancreas development and β-cell function, less is known about its regulation, particularly at the post-translational level. Previous reports indicated nuclear translocation of IPF1/PDX1 upon glucose stimulation that correlates with an increase in insulin-promoter activity (22, 23). Others have reported a nutrient-dependent regulation of insulin promoter activity through increased DNA binding capacity of IPF1/PDX1 (24–26). IPF1/PDX1 has also been shown to be potentially modified by post-translational mechanisms such as phosphorylation (24, 27, 28), glycosylation (29), and sumoylation (30). However, the exact post-translational modification involved, the conditions when this occurs, the underlying mechanism, and the effect on IPF1/PDX1 function remains largely unresolved.

Glycogen synthase kinase 3 (GSK3)3 is an ubiquitously expressed serine/threonine kinase that has been implicated in multiple processes including cell fate determination, metabolism, transcriptional and translational control, oncogenesis, and neurological diseases (31). There are two mammalian isoforms of GSK3 encoded by different genes, GSK3α and GSK3β, that are highly homologous and share substrate specificity in vitro. Unlike most protein kinases, GSK3 is constitutively active in resting cells and is inactivated after cellular stimulation by growth factors or hormones such as Wnt ligands and insulin. GSK3 phosphorylates a broad range of substrates including important growth regulators such as β-catenin, cyclin D1, and p21, as well as numerous transcription factors including c-Myc and c-Jun (31).

The current study was undertaken to clarify post-translational modifications involved in the modulation of the pancreatic transcription factor IPF1/PDX1 function. Herein, we demonstrate that IPF1/PDX1 can be phosphorylated in vivo on serine 61 and/or serine 66 in pancreatic β-cells. We also show that this phosphorylation targets the protein for degradation by the proteasome machinery and that it is GSK3-dependent. Finally, the present data reveal that oxidative stress, which occurs in the diabetic state, increases serine 61 and/or serine 66 phosphorylation, which correlates with an increased degradation rate and a decreased half-life for the IPF1/PDX1 protein. Hence, our data provide...
Evidence of a new mechanism that leads to reduced levels of IPF1/PDX1 protein and β-cell dysfunction during the development of diabetes.

**EXPERIMENTAL PROCEDURES**

**Materials**—Calf intestinal phosphatase (CIP) and Complete Mini protease inhibitor mixture tablets were obtained from Roche Applied Science. All other materials were obtained from Sigma unless stated otherwise.

**Antibodies**—For the production of the anti-phospho-IPF1/PDX1 antibody, a synthetic peptide covering the region 57LEQpSPPDipSPYE66 (Innovagen) of IPF1/PDX1, conjugated to keyhole limpet hemocyanin protein using a N-terminal cysteine, was used to immunize rabbits (Agrisera, Sweden). Antiserum was affinity-purified using the synthetic peptide conjugated to UltraLink agarose beads (Nordic Bios). Preparation of polyclonal anti-pan-IPF1/PDX1 antisem has been described previously (12). Anti-His monoclonal antibody was from Qiagen. Monoclonal antibody against GSK3β was purchased from Transduction Laboratories (BD Biosciences Pharmingen, Stockholm, Sweden). Polyclonal antibodies recognizing phosphorylation on serine 21 of GSK3α and serine 9 of GSK3β and ERK1/2 were obtained from Cell Signaling (In Vitro, Stockholm, Sweden).

**Expression Vectors and Constructs**—The full-length IPF1/PDX1 cDNA was subcloned into pcDNA3.1 mammalian expression vector, and a His, tag was added by PCR using an oligonucleotide containing the sequence encoding the tag. S61A, S61D, S66A, and S66D mutations were introduced into the pcDNA3.1-IPF1/PDX1 vector using a QuikChange site-directed mutagenesis (Stratagene) according to the manufacturer’s protocol. The insulin reporter construct used for luciferase assays contained the rat insulin promoter I (RIP) from residues 410 to +51 cloned upstream of the luciferase gene. All of the constructs generated by cloning and mutagenesis were confirmed by DNA sequencing. The mouse cDNA coding for GSK3β (pCMV-SPORT6-GSK3β) was from an image clone (GI:13905273).

**Islet Isolation and Cell Culture**—Islets were isolated by collagenase digestion of pancreas (32). Before treatment, the islets were incubated for 1 h at 37 °C in Dulbecco’s modified Eagle’s medium containing 3 mM D-glucose. For overnight cultures the islets were incubated at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 3 or 25 mM glucose. The islets were then lysed as described below or fixed with paraformaldehyde 4%, and frozen in Tissue Tek. The mouse αTC1/16, βTC3, and MIN6 cell lines were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 5.5 mM D-glucose and 2 mM glutamine supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin in humidified 5% CO2, 95% air at 37 °C. The human embryonic kidney 293 (HEK293) cells were cultured in Dulbecco’s modified Eagle’s medium containing 25 mM glucose, 2 mM glutamine, 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

**Immunohistochemistry**—Immunohistochemistry was carried out as described elsewhere (4). At least 20 unique islets were analyzed for each glucose concentration. Primary antibodies used were pan-IPF1/PDX1 (12) and phospho-IPF1/PDX1 antibody (this paper). Secondary antibodies used were: Cy3-anti-rabbit (Jackson Laboratory).

**Protein Expression and Immunoblotting**—The cells were washed twice with ice-cold PBS, lysed in Tritton buffer (1% Triton X-100, 50 mM Tris, pH 7.5, 100 mM NaCl, 5 mM EDTA, 0.2 mM orthovannadate, 40 mM β-glycerophosphate, 50 mM NaF, 10% glycerol, and 10% of protease inhibitor mixture) and cleared of cellular debris by centrifugation (10,000 rpm, 10 min, 4 °C). The protein concentrations were measured using BCA (Pierce) with bovine serum albumin as standard. Equal amounts of proteins were separated by SDS-PAGE (polyacrylamide gel electrophoresis), and the proteins were detected immunologically after electrotransfer onto nitrocellulose membranes. The membranes were blocked in PBS containing 5% nonfat dry milk and 0.05% Tween 20 for 1 h at 25 °C. The membranes were then incubated with appropriate primary antibodies in blocking solution followed by incubation with horseradish peroxidase-conjugated secondary antibodies. After extensive washing in PBS with 0.05% Tween, the blots were visualized with chemiluminescence reagent.

**Immunoprecipitation and Dephosphorylation of IPF1/PDX1 by CIP**—The cells were solubilized in Tritton buffer, and 1 mg of cleared lysate was immunoprecipitated with pan-IPF1/PDX1 antibody for 2 h at 4 °C. Protein G-Sepharose (Amersham Biosciences) was then added for 1 h at 4 °C. Protein G-Sepharose complexes were washed three times with Tritton buffer or assay buffer (for dephosphorylation assay) (1% Triton X-100, 50 mM Tris, pH 7.5, 100 mM NaCl, 0.5 mM EDTA, and 10% of protease inhibitor mixture). For dephosphorylation assay, the beads were equally split in two Eppendorf tubes. CIP (30 units/ml) was added to one of them, and both tubes were then incubated for 30 min at 30 °C. The reaction was stopped by adding 4× Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 2.2% SDS, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 5% β-mercaptoethanol, 0.005% bromophenol blue).

**Transient Transfections and Luciferase Assays**—The cells were seeded in 6-well plates (for protein expression analysis) or 24-well plates (for luciferase assay) and transfected with Lipofectamine (Invitrogen) according to the manufacturer’s protocol using the indicated amount of DNA. Empty expression plasmid was used to maintain a constant total amount of DNA among wells. For protein expression analysis, the cells were harvested 48 h after transfection, and protein lysates were prepared as described above. For luciferase assays, the cells were harvested 36 h after transfection with passive lysis buffer (Promega), and luciferase activities were determined using the dual luciferase assay kit (Promega) according to the manufacturer’s instructions. The data are expressed as firefly luciferase activity divided by Renilla luciferase (from pRL-CMV Renilla luciferase vector; Promega) activity to control for transfection efficiency among the different conditions.

**Data Presentation**—All of the experiments were performed at least in triplicate. Typical Western blots are representative of three independent experiments. Densitometric analyses were performed using the Scion Image 4.02 software package (Scion Corp., Frederick, MA).

**RESULTS**

**A Minor Portion of IPF1/PDX1 Is Phosphorylated in Pancreatic β-Cells**—In the pancreatic β-cell line, MIN6, immunoprecipitation followed by Western blot analysis using an anti-pan-IPF1/PDX1 antibody revealed two bands: a major band at ~42 kDa and a minor band of higher molecular mass (43 kDa) (Fig. 1A). Because phosphorylation has been proposed as a potential post-translational modification for IPF1/PDX1 (24, 27, 28), we hypothesized that the upper band might correspond to a phosphorylated form of IPF1/PDX1. To test this hypothesis, IPF1/PDX1 was immunoprecipitated and submitted to CIP treatment. Following CIP treatment, the 43-kDa band disappeared, and only the major 42-kDa band could be detected using the anti-pan-IPF1/PDX1 antibody (Fig. 1B), thus supporting the idea that the 43-kDa protein is a phosphorylated form of IPF1/PDX1. We next analyzed IPF1/PDX1 protein expression in mouse islets and in different pancreatic cell lines. The phosphorylated and nonphosphorylated forms of IPF1/PDX1 protein were present in islets and all pancreatic β-cell lines tested but not in the...
pancreatic α-cell line aTC1/16 (Fig. 1C), suggesting that a minor portion of IPF1/PDX1 is phosphorylated in pancreatic β-cells.

By examination of the N-terminal sequence, we identified two conserved potential phosphorylation sites, Ser61 and Ser66, that also have been suggested by others as potential phosphorylation sites for IPF1/PDX1 (20, 28). Therefore, we developed a specific antibody that recognizes phosphorylation of IPF1/PDX1 on Ser61 and/or Ser66 (see “Experimental Procedures”). By Western blot, the anti-phospho-IPF1/PDX1 antibody recognized only one band (Fig. 1C) that corresponded to the highest molecular mass band (43 kDa) visualized by the original IPF1/PDX1 antibody (12), hereafter denoted by the pan-IPF1/PDX1 antibody. To assess which phosphorylated serine was recognized by our antibody and to study an eventual functional importance of IPF1/PDX1 Ser61 and/or Ser66 phosphorylation, we proceeded to generate serine-to-alanine (to abolish phosphorylation) and serine-to-aspartic acid (to mimic phosphorylation) point mutations in the full-length Ipf1/Pdx1 coding sequence. His-tagged proteins were expressed in HEK293 cells, and the expression of the proteins, together with their phosphorylation status, was examined. The wild type (WT) construct generated a major protein band of 42 kDa together with a minor band of higher molecular mass (43 kDa), the latter being recognized by the anti-phospho-IPF1/PDX1 antibody (Fig. 1D). Interestingly, the S61A mutant displayed only one major 42-kDa band, and no band corresponding to the phosphorylated IPF1/PDX1 (43-kDa band) was detected, whereas the S61D mutant showed one major 43-kDa band that could also be detected by the anti-phospho-IPF1/PDX1 antibody (Fig. 1D). The S66A mutant still exhibited two bands of 42 and 43 kDa, but the S66D mutant was expressed as a single 43-kDa protein (Fig. 1D). In both cases the 43-kDa band was detected by the anti-phospho-IPF1/PDX1 antibody (Fig. 1D). Taken together, these results indicate that our anti-phospho-IPF1/PDX1 antibody can recognize either Ser61 or Ser66 phosphorylation on IPF1/PDX1 protein, that phosphorylation on Ser61 is critical to generate electrophoretic mobility shift of IPF1/PDX1, and that Ser61 phosphorylation is necessary to subsequently achieve Ser66 phosphorylation. Moreover, these results support our
findings that IPF1/PDX1 is phosphorylated in vivo on Ser\(^{61}\) and/or Ser\(^{66}\) in pancreatic \(\beta\)-cells and suggest that Ser\(^{61}\) and/or Ser\(^{66}\) are likely to be targets for kinases.

The subcellular localization of IPF1/PDX1 has been suggested to be regulated by glucose in a phosphorylation-dependent manner. At low concentrations, 0.5–3 mM glucose, IPF1/PDX1 was found to be unphosphorylated, excluded from the nucleus, and located in the cytoplasm, whereas shifting cells to glucose concentrations of 10 mM and higher, resulted in a complete translocation of IPF1/PDX1 to the nucleus (23, 33). Thus, we next proceeded to investigate the subcellular localization of IPF1/PDX1 using both the anti-pan-IPF1/PDX1 and the anti-phospho-IPF1/PDX1 antibodies. As expected IPF1/PDX1, detected using both the anti-pan-IPF1/PDX1 and anti-phospho-IPF1/PDX1 antibodies, was nuclear in islets and MIN6 cells cultured at high glucose concentrations (Fig. 1b and data not shown). However, also at low glucose conditions (3 mM), distinct nuclear staining was detected using both the anti-pan-IPF1/PDX1 and anti-phospho-IPF1/PDX1 antibodies (Fig. 1E and data not shown). Taken together, these data demonstrate that irrespective of the glucose concentrations, and phosphorylation state, the IPF1/PDX1 protein is nuclear.

**Accumulation of Phosphorylated IPF1/PDX1 Following Proteasome Inhibition**—There is accumulating evidence that the ubiquitin-proteasome system controls transcription factors at multiple levels and thus influences transcription (34). Moreover, it has recently been shown that MafA and IPF1/PDX1, two key players in insulin gene expression, are ubiquitinated and degraded in response to oxidative stress (35). Therefore, we next tested the effect of the proteasome inhibitor LLnL (36) on IPF1/PDX1 phosphorylation and protein levels. As shown in Fig. 2, treatment of cells with LLnL led to a distinct accumulation of phosphorylated IPF1/PDX1 protein (3.6-fold increase after 4 h and 9.1-fold increase after 8 h), whereas no significant change were observed in the nonphosphorylated IPF1/PDX1 protein level. Coincubation with the protein synthesis inhibitor cycloheximide did not prevent the LLnL-induced accumulation of phosphorylated IPF1/PDX1, indicating that this modification is post-translational (data not shown). This observation suggests that the phosphorylated form of IPF1/PDX1 is preferably targeted for degradation by the proteasome machinery.

**GSK3-dependent Mechanisms Modulate IPF1/PDX1 Phosphorylation**—In an attempt to investigate potential kinases involved in IPF1/PDX1 Ser\(^{61}\) and/or Ser\(^{66}\) phosphorylation, we treated MIN6 cells with different kinase inhibitors and analyzed IPF1/PDX1 phosphorylation status using our anti-phospho-IPF1/PDX1 antibody. No modulation of IPF1/PDX1 phosphorylation was found following treatment with the MEK inhibitor U0126, the calmodulin kinase inhibitor KN93, or the calcineurin inhibitor FK506 (data not shown), previously shown to influence mRNA expression of the IPF1/PDX1 target gene insulin (28, 37–39). However, treatment of MIN6 cells with the GSK3 inhibitor, LiCl (40), resulted in a slight but consistent reduction in IPF1/PDX1 phosphorylation by \(\sim 30\%\), without significantly affecting total IPF1/PDX1 protein expression (Fig. 3A).

**FIGURE 2.** Increased phosphorylation of IPF1/PDX1 observed following proteasome inhibition. MIN6 cells were cultured overnight in medium without serum containing 3 mM glucose prior to treatment with vehicle alone (Me\(_2\)SO) or 20 \(\mu\)M LLnL for the indicated periods. Equal amounts of whole cell lysates were separated by SDS-PAGE and submitted to Western blot analysis using the anti-phospho-IPF1/PDX1 and the anti-pan-IPF1/PDX1 antibodies.

**FIGURE 3.** GSK3-dependent modulation of IPF1/PDX1 phosphorylation. A, MIN6 cells were cultured overnight in medium without serum containing 3 mM glucose prior to treatment with 20 mM LiCl for the indicated periods. Equal amounts of whole cell lysates were separated by SDS-PAGE and submitted to Western blot analysis using the anti-phospho-IPF1/PDX1 and the anti-pan-IPF1/PDX1 antibodies. B, HeK293 cells were transiently transfected with equal amounts of DNA from pcDNA3-derived vector encoding His-tagged IPF1/PDX1 WT, together with expression vector for GSK3b or empty vector (–). 24 h after transfection, the cells were serum-starved. The cell lysates were prepared 48 h after transfection following a 4-h treatment with 20 mM NaCl (as control) or 20 mM LiCl. Equal amounts of whole cell lysates were separated by SDS-PAGE and submitted to Western blot analysis using the indicated antibodies. The blots shown are representative of four independent experiments. C, MIN6 cells were cultured overnight in medium without serum containing 3 mM glucose prior to treatment with vehicle alone (Me\(_2\)SO), 20 mM NaCl, or 20 mM LiCl or 20 \(\mu\)M LLnL for 4 h. Equal amounts of whole cell lysates were separated by SDS-PAGE and submitted to Western blot analysis using the anti-phospho-IPF1/PDX1 and the anti-pan-IPF1/PDX1 antibodies.
Interestingly, in IPF1/PDX1 WT-transfected HEK293 cells, LiCl treatment also reduced exogenous IPF1/PDX1 phosphorylation by 60% (Fig. 3B). Conversely, HEK293 cells cotransfected with IPF1/PDX1 WT and GSK3β showed an increase in IPF1/PDX1 phosphorylation (1.5-fold) compared with control cells transfected with IPF1/PDX1 alone. This level was comparable with the 1.9-fold increase in exogenous GSK3β protein expression. Moreover, the addition of LiCl greatly reduced the GSK3β-induced IPF1/PDX1 phosphorylation (Fig. 3B). Taken together, these results suggest that GSK3 activity is likely to influence IPF1/PDX1 phosphorylation on Ser61 and/or Ser66.

Because our results suggested that phosphorylation of IPF1/PDX1 might be a signal for degradation by the proteasome machinery, we next evaluated whether inhibition of GSK3 activity could prevent accumulation of the phosphorylated IPF1/PDX1 protein following proteasome inhibition. As shown in Fig. 3C, LiCl treatment prevented the LLnL-induced accumulation of phosphorylated IPF1/PDX1, supporting the idea that phosphorylation of either Ser61 and/or Ser66 of IPF1/PDX1 targets the phosphorylated protein for degradation by GSK3-dependent mechanisms.

**Oxidative Stress Induces IPF1/PDX1 Phosphorylation**—There is now considerable evidence that oxidative stress, which is found in the diabetic state, is involved in the progression of β-cell deterioration (41). To elucidate whether IPF1/PDX1 phosphorylation is affected by the oxidative state, we submitted cells to oxidative stress by treating them with H$_2$O$_2$. The addition of 50 μM H$_2$O$_2$ led within 30 min to a 2-fold induction of IPF1/PDX1 phosphorylation (Fig. 4A). Comparable results were obtained in isolated mouse islets (Fig. 4B). Interestingly, as shown in Fig. 5A, dose-response analysis of IPF1/PDX1 phosphorylation after H$_2$O$_2$ treatment revealed an even higher level of phosphorylated IPF1/PDX1 (nearly 4-fold increase compared with the 2-fold increase induced by 50 μM H$_2$O$_2$) following 2 h of treatment with 100 μM H$_2$O$_2$, which correlated with a 70% reduction in the nonphosphorylated IPF1/PDX1 protein expression. Because our data suggested that phosphorylation triggers IPF1/PDX1 protein for degradation, we then hypothesized that a sustained increase in IPF1/PDX1 phosphorylation will negatively affect the protein half-life. To confirm this, we evaluated IPF1/PDX1 protein stability in cells treated with H$_2$O$_2$, where protein synthesis has been inhibited by cycloheximide. As depicted in Fig. 5B, lower expression of IPF1/PDX1 was detected in cells treated with H$_2$O$_2$ + cycloheximide compared with cells treated alone with either H$_2$O$_2$ or cycloheximide. Quantification of the data (Fig. 5C) revealed that IPF1/PDX1 protein half-life was more than 8 h in H$_2$O$_2$ or cycloheximide-treated cells but was ~6 h in H$_2$O$_2$ + cycloheximide-treated cells, suggesting an increase in the degradation rate of IPF1/PDX1 following H$_2$O$_2$ treatment. As shown in Fig. 5D (and Fig. 2), only a slight accumulation of phosphorylated IPF1/PDX1 was observed in 2-h LLnL-treated cells (lane 1 versus lane 5 in Fig. 5D). However, in H$_2$O$_2$-treated cells, LLnL greatly increased the accumulation of phosphorylated IPF1/PDX1 (lane 7) compared with control cells (lanes 1 and 5) or H$_2$O$_2$-treated alone cells (lane 3), providing evidence that H$_2$O$_2$ treatment induces phosphorylation of IPF1/PDX1, which is then degraded by the proteasome. Taken together, these results suggest that oxidative stress, which leads to excessive and sustained phosphorylation of IPF1/PDX1, results in IPF1/PDX1 protein instability.

Recently, reports have shown an activation of GSK3 in response to different forms of cellular stress such as DNA damage and endoplasmic reticulum stress, suggesting that GSK3 might play a central role in signaling downstream effects of cellular stress (42–44). Our data provide evidence for a potential role for GSK3 in oxidative stress-mediated effects in β-cells. In our 100 μM H$_2$O$_2$-treated cells, we were able to detect a slight decrease in GSK3α S21 and GSK3β S9 phosphorylation, suggesting an increase in GSK3 activities in H$_2$O$_2$-treated cells (Fig. 5A). Also, in mouse islets (Fig. 4B) and in MIN6 cells (Fig. 5E), pretreatment with LiCl partially prevented the 30-min H$_2$O$_2$-induced IPF1/PDX1 phosphorylation. Finally, the addition of LiCl completely prevented the accumulation of phosphorylated IPF1/PDX1 in H$_2$O$_2$-treated cells following proteasome inhibition (Fig. 5D). So, to further test whether GSK3 was participating in oxidative stress-mediated IPF1/PDX1 protein instability, we evaluated the effect of lithium chloride on H$_2$O$_2$-induced IPF1/PDX1 protein degradation. Interestingly, as shown in Fig.
The addition of LiCl to H2O2-treated cells prevented the loss of IPF1/PDX1 protein expression, strongly supporting an important role for GSK3 activities in H2O2-induced IPF1/PDX1 degradation.

Ser61 and Ser66 Phosphorylation Do Not Modulate IPF1/PDX1 Activity—We next investigated whether IPF1/PDX1 Ser61 and/or Ser66 phosphorylation could modulate its transcriptional activity. First, using MIN6 cells, we assessed the effect of H2O2 on the insulin gene promoter activity. 50 mM H2O2 was sufficient to induce IPF1/PDX1 phosphorylation but only moderately affected IPF1/PDX1 protein levels, and no significant effect was observed with respect to insulin promoter activity. At 100 mM H2O2, IPF1/PDX1 phosphorylation was further increased and paralleled by considerably reduced levels of IPF1/PDX1 protein, whereas no significant effect was observed with respect to transcriptional activity (Fig. 6A). 8 h of treatment with 100 mM H2O2 resulted, however, in slightly reduced insulin promoter activity, probably as a consequence of reduced levels of IPF1/PDX1 protein. We did not observe any modulation of IPF1/PDX1-induced insulin promoter activity following LiCl treatment or GSK3β overexpression (data not shown).

Using our IPF1/PDX1 mutants, which reflect different levels of IPF1/PDX1 Ser61 and Ser66 phosphorylation (Fig. 1D), we were also unable to detect any significant differences in IPF1/PDX1-induced insulin promoter activity (Fig. 6B). We next investigate whether the synergetic effect reported for IPF1/PDX1 and its coactivators on insulin promoter activity would be affected by our mutants. We did observe a synergetic effect using E2A family proteins on insulin promoter activity; however,
Regulation of IPF1/PDX1 Stability by Phosphorylation

Regulation of protein expression often implies post-translational modifications. In the present study, we demonstrate that IPF1/PDX1 protein can be phosphorylated at several sites, one of which is phosphorylated in a GSK3-dependent manner. This phosphorylation targets the protein for degradation by the proteasome machinery. However, in the current study, we cannot rule out the possibility of an indirect GSK3-dependent phosphorylation of IPF1/PDX1.

Herein, we observed a partial inhibition of IPF1/PDX1 phosphorylation by LiCl and/or Ser66 phosphorylation by LiCl after 30 min of treatment with H2O2. Because our anti-phospho-IPF1/PDX antibody is able to recognize both Ser61 and Ser66 phosphorylation, the partial effect of LiCl might reflect the inhibition of phosphorylation on only one serine. After 2 h of treatment with H2O2, the effect of LiCl on H2O2-induced Ser61 and/or Ser66 phosphorylation was hardly detectable. One explanation might be that the double (or more) phosphorylated form of IPF1/PDX1 might already have been targeted for degradation, and thus we only detect the incompletely phosphorylated IPF1/PDX1 protein in both H2O2-treated and LiCl-treated cells.

In accordance with this, we did not detect increased IPF1/PDX1 phosphorylation in H2O2 + LiCl-treated cells. In contrast, in the current study, we cannot rule out the possibility of an indirect GSK3-dependent phosphorylation of IPF1/PDX1.

Another novel and interesting finding from our work is the demonstration that GSK3-dependent mechanisms modulate Ser61 and/or Ser66 phosphorylation of IPF1/PDX1. As mentioned above, phosphorylation of those serine residues seems to critically affect IPF1/PDX1 protein stability. It is noteworthy that GSK3 has been involved in the protein stability of other proteins implicated in the regulation of transcription, including for instance β-catenin (46, 47), cyclin D1 (48), c-Myc (49), and, more recently, c-Jun (50), NAC (51), mCRY2 (52), and p53 (53). Except for p53, it was demonstrated that direct phosphorylation by GSK3 targets the protein for degradation by the proteasome machinery. However, in the current study, we cannot rule out the possibility of an indirect GSK3-dependent phosphorylation of IPF1/PDX1.

FIGURE 6. Ser61 and Ser66 phosphorylation do not modulate IPF1/PDX1 transcriptional activity. A, MIN6 cells were transfected with 0.1 μg of RIP luciferase and 0.01 μg of Renilla luciferase activity divided by g of RIP luciferase and 0.01 μg of Renilla luciferase activity. B, HEK293 cells were transfected with 0.1 μg of empty vector (pcDNA3) or pcDNA3-derived vectors encoding either WT or mutants IPF1/PDX1, 0.1 μg of RIP luciferase and 0.01 μg of Renilla luciferase. The cells were lysed, and luciferase activity was measured 36 h after transfection. The data are expressed as RIP luciferase activity divided by Renilla luciferase activity where relative activity in untreated cells was set at 100%. The results are the means ± S.E. of three separate experiments done in triplicate. *, significantly different from control (CTL) at p < 0.001 (Student’s t test).

this response was similar for the IPF1/PDX1 WT and the different mutants (data not shown). Taken together, these results suggest that Ser61 and/or Ser66 phosphorylation do not per se influence IPF1/PDX1 transcriptional activity.

DISCUSSION

The IPF1/PDX1 transcription factor plays a crucial role in pancreas development and β-cell function. Despite a number of studies delineating the molecular mechanisms controlling IPF1/PDX1 mRNA expression, not much has been done to define molecular mechanisms regulating IPF1/PDX1 expression at the protein level. Regulation of protein expression often implies post-translational modifications. In the present study, we demonstrate that IPF1/PDX1 protein can be phosphorylated in vivo in pancreatic β-cells and that this phosphorylation of IPF1/PDX1 marks the protein for degradation by the proteasome machinery.

We have identified two serine residues (Ser61 and Ser66), located in the transactivation domain of IPF1/PDX1 and conserved among different species, that can be phosphorylated in vivo in pancreatic β-cells. Our results suggest that phosphorylation on Ser61 is an important event triggering subsequent Ser66 phosphorylation of IPF1/PDX1. Indeed, abolition of Ser61 phosphorylation (i.e. S61A mutation) prevented the electrophoretic mobility shift (reflecting phosphorylation of the protein) and Ser66 phosphorylation, whereas mimicking Ser61 phosphorylation (i.e. the S61D mutation) was sufficient to promote electrophoretic mobility shift and Ser66 phosphorylation. However, our data do not exclude that Ser61 and, subsequently, Ser66 phosphorylation might also promote phosphorylation of the protein on other sites. In this regard, when resolution of the SDS-PAGE was optimal, we were able to detect, using the anti-phospho-IPF1/PDX1 antibody, at least three different bands (see IPF1/PDX1 WT phosphorylation status on Fig. 1D), suggesting several levels of phosphorylation on IPF1/PDX1 protein. Moreover, it seems to be the highest molecular mass phosphorylated IPF1/PDX1 that accumulates in LLNL-treated cells. During the preparation of this paper, another study reported that phosphorylation of IPF1/PDX1 on threonine 11 correlates with degradation of the phosphorylated protein by the proteasome (45). Thus, sequential phosphorylation events might be needed to ultimately target IPF1/PDX1 for degradation.

The data presented herein suggest that phosphorylation of Ser61 and/or Ser66 decreases IPF1/PDX1 protein stability given that (i) preferential accumulation of phosphorylated IPF1/PDX1 (on Ser61 and/or Ser66) was observed following proteasome inhibition, (ii) blockade of IPF1/PDX1 phosphorylation by LiCl prevented both the accumulation of the phosphorylated protein following proteasome inhibition and the degradation of IPF1/PDX1, and (iii) decreased protein level expression, increased degradation rate, and decreased half-life of IPF1/PDX1 protein were observed under condition where excessive and sustained IPF1/PDX1 phosphorylation on Ser61 and/or Ser66 was monitored.

Another novel and interesting finding from our work is the demonstration that GSK3-dependent mechanisms modulate Ser61 and/or Ser66 phosphorylation of IPF1/PDX1. As mentioned above, phosphorylation of those serine residues seems to critically affect IPF1/PDX1 protein stability. It is noteworthy that GSK3 has been involved in the protein stability of other proteins implicated in the regulation of transcription, including for instance β-catenin (46, 47), cyclin D1 (48), c-Myc (49), and, more recently, c-Jun (50), NAC (51), mCRY2 (52), and p53 (53). Except for p53, it was demonstrated that direct phosphorylation by GSK3 targets the protein for degradation by the proteasome machinery. However, in the current study, we cannot rule out the possibility of an indirect GSK3-dependent phosphorylation of IPF1/PDX1.

Herein, we observed a partial inhibition of IPF1/PDX1 phosphorylation by LiCl and/or Ser66 phosphorylation by LiCl after 30 min of treatment with H2O2. Because our anti-phospho-IPF1/PDX antibody is able to recognize both Ser61 and Ser66 phosphorylation, the partial effect of LiCl might reflect the inhibition of phosphorylation on only one serine. After 2 h of treatment with H2O2, the effect of LiCl on H2O2-induced Ser61 and/or Ser66 phosphorylation was hardly detectable. One explanation might be that the double (or more) phosphorylated form of IPF1/PDX1 might already have been targeted for degradation, and thus we only detect the incompletely phosphorylated IPF1/PDX1 protein in both H2O2-treated and LiCl-treated cells. In accordance with this, we did not detect increased IPF1/PDX1 phosphorylation in H2O2 + LiCl-treated cells. In contrast, in the current study, we cannot rule out the possibility of an indirect GSK3-dependent phosphorylation of IPF1/PDX1.

A number of studies have shown that c-Jun (50) and c-Myc (53) are phosphorylated on several sites, one of which is phosphorylated in a GSK3-dependent manner. These studies show that both the GSK3-dependent phosphorylated site and the phosphorylated priming site are needed for the recognition and destruction of the transcription factor by the Fbw7 ubiquitin ligase. Thus, a similar mechanism could be proposed for IPF1/PDX1, where blockade of one phosphorylation site by GSK3 inhibition prevents the recognition of IPF1/PDX1 by an ubiquitin ligase, i.e. the ligase necessary to target the protein for degradation. Additional experiments will be required to clarify the role of GSK3 on IPF1/PDX1 phosphorylation and protein stability.
Regulation of IPF1/PDX1 Stability by Phosphorylation

GSK3 is an attractive candidate for regulation of IPF1/PDX1 protein stability given that a role for GSK3 in type II diabetes is now well recognized. Type II diabetes is characterized by the inability of peripheral tissues (such as muscle, liver, and fat) to respond normally to physiological concentrations of insulin but also by β-cell dysfunction. Interestingly, an elevated level of GSK3 activity has been observed in insulin target tissues of diabetic mice and human (55, 56). Moreover, GSK3 inhibitors, including lithium chloride, have been shown to improve glucose metabolism in insulin-resistant Zucker rats (57, 58). However, in those studies, a potential role for GSK3 activity in diabetic β-cell metabolism in insulin-resistant Zucker rats (57, 58) has not been addressed. Another study indirectly implies a relation between GSK3 activity and β-cell function. Active canonical Wnt signaling leads to inhibition of GSK3 activity and islets isolated from 6-month-old, but not younger, mice lacking the Wnt coreceptor LRPs5, which transduce canonical Wnt signals (59), showed impaired glucose-induced insulin secretion (60). Oxidative stress increases with age, and β-cells are sensitive to oxidative stress, thus leaving open a possible scenario where canonical Wnt signaling, by suppressing GSK3 activity, counteracts age-related increases in oxidative stress, thereby preventing enhanced IPF1/PDX1 protein degradation and consequently assuring maintenance of β-cell function.

After the onset of type II diabetes, hyperglycemia arises, and glucose in chronic excess ultimately leads to toxic effects on structure and function of several organs including the pancreatic β-cells (41). Multiple biochemical pathways and mechanisms of action for glucose toxicity have been suggested; however, all of these pathways have in common the formation of reactive oxygen species that, in excess and over time, causes chronic oxidative stress. In the present study, we have demonstrated that peroxide hydrogen increases IPF1/PDX1 Ser41 and/or Ser66 phosphorylation, which correlates with an increased degradation rate and a decreased half-life for IPF1/PDX1 protein. Previous studies have shown a negative impact of oxidative stress on insulin gene expression and insulin secretion. To explain these effects, nucleo-cytoplasmic translocation of IPF1/PDX1 has been proposed (61). However, the experiment was undertaken after 48 h of treatment with 50 μM H2O2. In our hands, we did not observe any change in IPF1/PDX1 subcellular localization after 1 or 4 h of treatment with H2O2 (data not shown), and we did not look at later stages because prolonged treatment with 50 μM H2O2 (more than 8 h) begins to affect cell viability, which could influence the interpretation of the results. Others have shown a reduction in DNA-binding of IPF1/PDX1 to the insulin promoter after 48 h of treatment with H2O2 (62) or after the addition of d-ribose for 5 days (63). However, IPF1/PDX1 mRNA and protein expressions were not evaluated, and decreased DNA binding activity might have been the result of decreased IPF1/PDX1 protein levels. In the present study, no modulation in insulin promoter activity was detected after short exposure of H2O2, but reduced IPF1/PDX1 protein expression was observed after prolonged H2O2 treatment. Thus, it seems probable that prolonged oxidative stress might lead to reduced IPF1/PDX1 protein levels, thereby affecting transcription of IPF1/PDX1 target genes. Interestingly, diabetic mice fed a high antioxidant diet showed a stronger IPF1/PDX1 β-cell immunoreactivity compared with that observed in diabetic mice on a regular diet (64). These results are in agreement with our current hypothesis of a negative regulation of IPF1/PDX1 protein stability by oxidative stress.

Interestingly, recent studies have demonstrated an activation of GSK3 under stress conditions. DNA damage (42) and endoplasmic reticulum stress (43) have been shown to induce both GSK3 activity and nuclear localization of the kinase. Moreover, valproate has been shown to protect cells from endoplasmic stress-induced lipid accumulation and apoptosis by inhibiting GSK3 activity (44). Thus, there is accumulating evidence in the literature demonstrating a potential role for GSK3 in cellular stress-mediated effects. In the present study, we have demonstrated that GSK3 activity plays a role in oxidative stress-induced IPF1/PDX1 degradation. Hence, it would be interesting to test, in the context of diabetes progression, if maintenance of a low GSK3 activity in β-cells protects IPF1/PDX1 protein from degradation and thereby helps β-cells to assure their function.

In conclusion, we provide evidence that a minor portion of IPF1/PDX1 is phosphorylated in β-cells that probably reflect the basal turnover of the protein. If increased and sustained phosphorylation of IPF1/PDX1 occurs, as for example during oxidative stress, de novo protein synthesis might be insufficient to overcome the loss of IPF1/PDX1 protein because of degradation, and consequently a reduction in IPF1/PDX1 protein levels is observed. Because reduced levels of expression of IPF1/PDX1 have been shown to influence insulin expression and secretion as well as β-cell survival (4–6), it seems likely that chronic oxidative stress, by decreasing IPF1/PDX1 protein expression, contributes to β-cell dysfunction. Finally, because GSK3 inhibitors are under investigation as potential treatment for diabetes (65, 66), it would be interesting to evaluate the effect of those compounds not only on glucose uptake by the peripheral tissues but also on β-cell function, i.e. insulin expression and secretion.

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