Pancreatic and duodenal homeobox 1 (PDX1) phosphorylation at serine-269 is HIPK2-dependent and affects PDX1 subnuclear localization

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ABSTRACT

Pancreatic and duodenal homeobox 1 (PDX1) regulates pancreatic development and mature β-cell function. We demonstrate by mass spectrometry that serine residue at position 269 in the C-terminal domain of PDX1 is phosphorylated in β-cells. Besides we show that the degree of phosphorylation, assessed with a phospho-Ser-269-specific antibody, is decreased by elevated glucose concentrations in both MIN6 β-cells and primary mouse pancreatic islets. Homeodomain interacting protein kinase 2 (HIPK2) phosphorylates PDX1 *in vitro*; phosphate incorporation substantially decreases in PDX1 S269A mutant. Silencing of HIPK2 led to a 51 ± 0.2% decrease in Ser-269 phosphorylation in MIN6 β-cells. Mutation of Ser-269 to phosphomimetic residue glutamic acid (S269E) or de-phosphomimetic residue alanine (S269A) exerted no effect on PDX1 half-life. Instead, PDX1 S269E mutant displayed abnormal changes in subnuclear localization in response to high glucose. Our results suggest that HIPK2-mediated phosphorylation of PDX1 at Ser-269 might be a regulatory mechanism connecting signals generated by changes in extracellular glucose concentration to downstream effectors via changes in subnuclear localization of PDX1, thereby influencing islet cell differentiation and function.

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1. Introduction

Control by glucose of preproinsulin gene transcription in pancreatic islet β-cells involves the interplay of multiple *trans*- and *cis*-activating factors ([1] and references therein). Pancreatic and duodenal homeobox 1 (PDX1) was first described as a preproinsulin promoter A3-site binding factor [2,3]. PDX1 is involved in both the development and function of pancreatic β and other islet cells [4].

Deletion or inactivation of the *PDX1* gene in the mouse [5] or in man [6] results in the complete failure of normal pancreatic development. Moreover, β-cell selective disruption of murine PDX1 expression [7] or function [8] lead respectively to defective insulin secretion and glucose signaling. On the other hand, heterozygosity for a defective *PDX1* gene leads to defective preproinsulin gene expression [7] and abnormal insulin secretion [9] in mice and to maturity-onset-diabetes of the young 4 (MODY4) in man [6].

In contrast to the relatively well-studied functions of the N-terminus and the homeodomain of PDX1, the role of the conserved C-terminus is less well defined. Mutations which affect the C-terminus of PDX1 are associated with the development of type 2 diabetes in humans [10–12], while other findings indicate that the C-terminal domain may serve as both repressor and activator of PDX1 function [13,14].

Humphrey and colleagues [15] reported that PDX1 phosphorylation in primary rat islets is decreased by high glucose levels. These authors described Ser-268 and Ser-272 of rat PDX1 (corresponding to Ser-269 and Ser-273 of mouse PDX1) as a novel C-terminal atypical non-primed GSK-3 consensus site which regulates PDX1 protein stability in response to glucose. Importantly, homeodomain interacting protein kinase 2 (HIPK2) ([16] and references therein) has been shown to co-localize with PDX1 in both the developing and adult pancreas and to modulate positively PDX1 transcriptional activity, possibly by phosphorylation of the C-terminal domain [17]. We have previously observed that, in clonal β-cells, elevated glucose concentrations lead to translocation of PDX1 between the nuclear periphery and the nucleoplasm, accompanied by increased preproinsulin promoter activity [18]. Although the molecular basis for the enhanced nucleoplasmic accumulation of PDX1 is unclear, this process may involve interaction of PDX1 homeodomain with the nuclear import receptor family member importin-β1 [19]. In the present study we used mass spectrometry and generated an anti-phospho-serine-specific antibody to confirm Ser-269 as a *bona*

fide phosphorylation site in mouse PDX1 that is regulated by glucose in MIN6 β-cells and in primary mouse islets of Langerhans. We show that Ser-269 is phosphorylated by homeodomain interacting protein kinase 2 (HIPK2) *in vitro*. The analysis of (de)phospho-Ser-269-specific mutants suggest that phosphorylation at this site, whilst having no effect on PDX1 protein stability or PDX1 DNA-binding property, is involved in nucleoplasmic (versus nuclear-peripheric) localization in the β-cell in response to glucose.

2. Materials and methods

The work described in this article has been carried out in accordance with the *EC Directive 86/609/EEC for animal experiments* http://europa.eu.int/scadplus/leg/en/s23000.htm; and the *Uniform Requirements for manuscripts submitted to Biomedical journals* http://www.nejm.org/general/text/requirements/1.htm.

2.1. Cell culture and reagents

Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Lonza) containing 10% (v/v) fetal bovine serum (FBS), 100 IU/ml penicillin and 100 IU/ml streptomycin. INS-1(832/13) cells (kindly provided by Dr. C. Newgard, Duke University) and MIN6 β (mouse insulinoma pancreatic beta) cells were cultured as in [20].

Anti-*c-myc* antibody was from Roche. Rabbit polyclonal anti-PDX1 antibody was as described [18]. Anti-phospho-Ser-269-PDX1 antibody was raised in rabbits by immunization with synthetic phospho-peptide: L^{262}PSGLSV**pS**PQPSSIAPLRPQEPR^{284} (Pacific Immunology Inc, USA). HIPK2 was purchased from Upstate (Lake Placid, NY).

2.2. Mouse islet isolation and culture

Islets were isolated from CD1 mice and cultured as previously described [21].

2.3. Plasmids

Plasmid pcDNA3-PDX1-*c-myc* has been described [18]. Mutant plasmids pcDNA3-PDX1-S269A-*c-myc* and pcDNA3-PDX1-S269E-*c-myc* were generated using a QuikChange site-directed mutagenesis kit (Stratagene). Wild-type and mutant PDX1 myc-tagged coding sequences were inserted (*Hind*III/*Eco*RV) into the shuttle vector pAd-Track-CMV multiple cloning site [22]. Mouse wild-type PDX1 sequence PCR amplified from plasmid pcDNA3-PDX1-*c-myc* was cloned (*Nco*I/*Bam*HI) into 6xHis-MBP (maltose binding protein) plasmid. Mutant plasmid His-MBP-PDX1 (S269A) was generated as above.

2.4. His-MBP-PDX1 production and purification

His-MBP-PDX1 proteins expression was induced in *Escherichia coli* BL21 with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Proteins were purified on a nickel–nitrilotriacetic acid column according to Qiagen and dialyzed for 16 h at 4 °C in 50 mM Tris pH 7.9, 150 mM NaCl, 5 mM MgCl$_2$, 1 mM β-mercaptoethanol. The MBP moiety was cut with Tobacco Etch Virus (TEV) protease, AcTEV^{TM} protease (Invitrogen). MBP, histidine tag and histidine-tagged Ac-TEV protease were removed respectively with Amylose beads (New England BioLab) and Ni–NTA agarose beads.

2.5. Recombinant adenoviruses and viral infection

Recombinant adenoviruses expressing wild-type (WT) and mutant (S269A, S269E) PDX1 and control adenovirus, expressing green fluorescent protein (Ad-GFP) were prepared using the AdEasy system [22]. Cells were infected with various adenoviruses at a multiplicity of infection (MOI) of 50 for 5 h and maintained in 25 mM glucose for 24 h before subsequent experiments.

2.6. Real-time RT-PCR

Total mRNA and real-time quantitative RT-PCR analysis was as [23]. Primer sequences are as follows: cyclophilin A fwd, 5′-TAT CTG CAC TGC CAA GAC TGA-3′; cyclophilin A rev, 5′-CCA CAA TGC TCA TGC CTT CTT TCA-3′; HIPK2 fwd, 5′-TGC TTG ACT TCC CCC ATA GTG -3′; HIPK2 rev, 5′-CTT GCA AAT CTC CAT GTT TTG G -3′.

Data were analyzed by ABR PRISM SDS v1.3.1 (Applied Biosystems).

2.7. Immunocytochemistry

MIN6 β-cells infected with wild-type or mutant forms of PDX1 viruses at 50 MOI (multiplicity of infection). Immunocytochemical analysis was performed as described in the figure legends and in [20].

2.8. Immunoprecipitation

Cells lysed on ice with 750 μl of IP buffer [1% (v/v) NP40 (Nonidet P40); 50 mM NaCl; 1% (w/v) sodium deoxycholate; 0.1% (w/v) sodium monododecyl sulfate (SDS); 50 mM Tris–HCl pH 7.5; 2 mM EDTA; 10 mM sodium phosphate; 50 mM NaF (sodium fluoride); 1 m PMSF; 200 μM Na$_3$VO$_4$ (sodium orthovanadate); 1 × Complete^{TM} protease inhibitor cocktail solution (Roche) and 1 × phosphatase inhibitor cocktail 1 & 2 (Sigma)] were rotated on wheel at 4 °C for 30 min. The lysate was clarified by centrifugation at 16,000g for 5 min. *c-myc* antibody-conjugated beads (Santa-Cruz; 40 μl) were added and tubes rotated at 4 °C overnight. After centrifugation at 1,000g at 4 °C for 30 s, beads were washed five times with lysis buffer.

2.9. In vitro phosphorylation by HIPK2

Purified wild-type (WT) or serine to alanine mutant (S269A) PDX1 (2 μg) was subjected to *in vitro* phosphorylation with HIPK2 (Upstate) following the manufacturer's instruction.

2.10. Western blotting

Nuclear proteins were extracted as in [24]. Protein samples were separated on SDS–PAGE, and analyzed by Western blotting with the indicated antibodies.

2.11. Silencing of HIPK2

siRNA were made with the AMBION Silencer siRNA construction kit. Starting primer pairs were designed as suggested in the AMBION protocol and according to the guidelines published in [25]. Sense and anti-sense primers were derived from mouse cDNA sequence for HIPK2 in the ENSEMBL database (transcript name: Hipk2-201; transcript Id: ENSMUST00000038777). Target sequence was from nucleotide 788 to nucleotide 808 as follows: 5′-AAA CGG GGC ACC AAT GAA ATT CCT GTC TC-3′). Control siRNA were made with primers derived by scrambling the siRNA sequence for HIPK2. All sequences were searched against known mouse cDNA sequences using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Transfection was with TransIT-TKO transfection reagent (Mirus Bio Corporation).

2.12. Pulse-chase

MIN6 β-cells were infected with PDX1 wild-type, S269A and S269E adenoviruses. 24 h after infection, media were replaced with