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Quantitative proteomics reveals novel protein interaction partners of PP2A catalytic subunit in pancreatic β -cells

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Abstract

Protein phosphatase 2A (PP2A) is one of the major serine/threonine phosphatases. We hypothesize that PP2A regulates signaling cascades in pancreatic β -cells in the context of glucose-stimulated insulin secretion (GSIS). Using co-immunoprecipitation (co-IP) and tandem mass spectrometry, we globally identified the protein interaction partners of the PP2A catalytic subunit (PP2Ac) in insulin-secreting pancreatic β -cells. Among the 514 identified PP2Ac interaction partners, 476 were novel. This represents the first global view of PP2Ac protein-protein interactions caused by hyperglycemic conditions. Additionally, numerous PP2Ac partners were found involved in a variety of signaling pathways in the β -cell function, such as insulin secretion. Our data suggest that PP2A interacts with various signaling proteins necessary for physiological insulin secretion as well as signaling proteins known to regulate cell dysfunction and apoptosis in the pancreatic β -cells.

Keywords

Quantitative proteomics; serine/threonine protein phosphatase 2A (PP2A); interaction partner; pancreatic β -cell; glucotoxic condition

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Author contributions

X.Z. and D.D. performed biological and proteomics experiments, analyzed data, generated figures, and wrote the manuscript. D.M. and Y.Q. performed proteomics experiments; R.T. and S.D. performed data analysis, A.K. designed the biological experiments, analyzed data, and wrote the manuscript; Z.Y. supervised the project, designed the biological and proteomic experiments, analyzed data, and wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.

1. Introduction

Glucose-stimulated insulin secretion (GSIS) from the pancreatic islet β -cell is a multifactorial process. It involves generation of second messengers, including cyclic nucleotides, adenine nucleotides, guanine nucleotides and lipid hydrolytic products of phospholipases A₂, C and D [1, 2]. Increase in intracellular calcium concentrations and consequential opening of voltage gated calcium channels have also been implicated in GSIS, primarily at the level of movement of insulin-laden secretory granules to the plasma membrane for fusion and exocytotic secretion of insulin. Some known actions of these second messengers include regulation of various protein kinases indigenous to pancreatic β -cells, including calcium-, calcium/calmodulin-, cAMP- and phospholipid-dependent protein kinases[3]. It is well established that the phosphorylation status of proteins is tightly regulated by the balance of the activities of protein kinases and phosphatases. Although several earlier studies were focused on the identification and characterization of protein kinases, roles of protein phosphatases in regulation of islet β -cell survival and function remain poorly understood [4, 5]. In context of protein phosphatases, original studies from our laboratory have suggested novel roles for okadaic acid-sensitive protein phosphatase 2A (PP2A) in GSIS[4].

The PP2A family of phosphatases represents a major class of phospho-serine/threonine phosphatases, which have been implicated in the regulation of many cellular events, including cell cycle progression, proliferation, cytoskeletal remodeling, hormone secretion, and even cellular dysfunction [4, 6, 7]. Several holoenzyme complexes of PP2A have been isolated and characterized from a variety of tissues. The PP2A heterodimer complex is comprised of a scaffolding A subunit with an apparent molecular mass of 65 kDa and the 36-kDa catalytic subunit (PP2Ac). This A/C subunit heterodimer interacts with a regulatory B subunit yielding the PP2A heterotrimeric holoenzyme. Two different genes of A (A α and A β) and C (C α and C β), and several families of the B subunits (B, B', B'', and B'''), have been identified. The binding of B subunit to the A/C heterodimer provides further stability to the holoenzyme. It is also suggested that the variable B subunit(s) influences substrate specificity and/or subcellular localization of a given PP2A holoenzyme [4, 6, 7]. It is estimated that the combination of all subunits (A, B, and C) could produce more than 75 different trimeric holoenzymes, the precise number of the possible holoenzyme complexes that actually exist in β -cells still needs to be determined. Although the A and C subunits are ubiquitously expressed, certain B subunits are expressed in a tissue-specific manner and at various stages of cellular development.

As noted above, interactions of PP2Ac with other key regulatory proteins/factors modulate PP2A holoenzyme activity and specificity. In addition, other proteins (e.g., substrates or regulators of PP2A) also interact with the PP2A holoenzyme, thereby promoting specific signaling pathways. PP2A has been shown to inactivate a large number of kinases (e.g. Akt, AMPK, PKC, p70S6 kinase) and activate other kinases (e.g. GSK-3, casein kinase I)[8, 9]. In addition, PP2Ac was identified as an interaction partner of insulin receptor substrate-1 (IRS1) in murine HL-1 cardiomyocytes[10]. More recently, we have demonstrated that PP2Ac interacted with IRS1 in skeletal muscle of lean healthy controls, and this interaction is increased in insulin resistant obese nondiabetic controls and type 2 diabetic patients [11].

It is well established that chronic exposure of pancreatic β -cells to high glucose (HG; glucotoxicity) leads to metabolic dysfunction and demise [12]. Several intracellular signaling events have been identified as causal to HG-induced metabolic dysregulation of the islet including endoplasmic reticulum stress, mitochondrial dysregulation and the collapse of nuclear compartment[13, 14]. Despite this compelling evidence, little is known about potential detrimental effects of HG on phosphatase function in the islet β -cell. Along these lines, we recently reported sustained activation of PP2A in normal rat islets and insulin-secreting INS-1 832/13 cells under HG conditions. We demonstrated that siRNA-mediated depletion of endogenous PP2Ac markedly attenuated HG-induced activation of PP2A. Based on these findings, we concluded that exposure of the islet β -cell to HG leads to accelerated PP2A signaling pathway, leading to loss in GSIS[15]. Despite this evidence, potential identity and involvement of interactions between PP2Ac and other proteins/factors in islet β -cells under HG exposure remain an important area of investigation.

Co-immunoprecipitation (co-IP) followed by mass spectrometry based proteomics have emerged as a powerful approaches to map protein-protein interactions[16]. However, most of these studies used protein overexpression and/or epitope-tagged bait proteins. Recently, we developed a straightforward, label-free approach combining co-IP with HPLC-ESI-MS/MS, without the use of protein overexpression or protein tags, to investigate changes in the abundance of endogenous proteins associated with a bait protein[17]. More recently, we have optimized this label-free approach, and discovered novel IRS-1 interaction partners in skeletal muscle from lean healthy, obese non-diabetic, and type 2 diabetic patients[11]. In the present work, we applied this proteomics approach to identify protein interaction partners of PP2Ac in pancreatic islet β -cells exposed to basal (2.5 mM) and glucotoxic (25 mM) conditions. The goal of the study is to identify the novel proteins interacting with PP2Ac in INS-1 832/13 cells. These studies are also aimed at determining potential alterations in protein-protein interactions involving PP2Ac in islet β -cells following exposure to glucotoxic condition, which has been shown to promote cellular dysregulation and apoptosis in these cells.

2. Materials and methods

2.1. Experimental design

As illustrated in Fig. 1, the present study started with cell culture of insulin-secreting β -cell line INS-1 832/13 under basal (2.5 mM) and glucotoxic (25 mM) conditions. We have reported significant impairment in GSIS in INS 832/13 cells under these conditions suggesting the utility of this *in vitro* model system for studies islet β -cell dysfunction [15]. The proteomics studies were conducted as follows: cell lysis, immunoprecipitation of the “bait” protein with mouse monoclonal PP2Ac antibody, at the endogenous level; followed by one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1D-SDS-PAGE) to separate co-interaction proteins; in-gel trypsin digestion to generate peptide fragments; and high performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) analysis to identify co-immunoprecipitated proteins. Multiple biological comparisons and immunoprecipitation of normal mouse IgG (NIgG, as non-specific control) were employed to minimize false positives. Extensive

bioinformatics and literature searches were conducted to integrate clinical and proteomics data and to identify pathways/functional categories, in which identified PP2Ac interaction partners were involved, that were impacted by HG treatment.

2.2. Cell culture and HG treatment

Insulin-secreting INS-1 832/13 cells were maintained in RPMI 1640 medium containing 11 mM glucose, 10% heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin, 100 IU/ml streptomycin, 1 mM sodium pyruvate, 50 μ M 2-mercaptoethanol and 10 mM HEPES (pH 7.4). To be treated with basal or high levels of glucose, the cells were first starved overnight in a medium which is similar as the above growth medium but contains 2.5 mM glucose and 2% FBS. For next 48 hrs, the cells were cultured in media which are similar as the growth medium but contain 2.5 mM or 25 mM glucose. The treated cells were harvested and homogenized in 1 ml of lysis buffer (2 mM EDTA, 2 mM EGTA, 20 mM imidazole-HCl, pH 7.0 with 1 mM PMSF, 10 μ g/mL leupeptin, and 10 μ g/mL aprotinin). After centrifugation of cell lysates, total protein was quantified by Bradford method. For each sample, 4 mg of total protein was first incubated with 4 μ g of normal mouse IgG (Millipore, Cat. No. 12–371) conjugated to protein A beads. Three hours later, beads were harvested and served as nonspecific control. The precleared supernatant was further incubated with 4 μ g of PP2Ac mouse monoclonal antibody (Millipore, Cat. No. 05–421) conjugated to protein A beads. After overnight incubation, the beads were harvested.

2.3. Proteomics sample preparation and analysis

Both NIGG and anti-PP2Ac beads were washed three times with PBS. Subsequently, the beads were boiled in 30 μ l of 2 $\times$ SDS buffer containing 50 mM dithiothreitol (DTT) at 95°C for 5 min, followed by iodoacetamide (IAA) treatment. Bead eluates were resolved by 4–15% SDS-PAGE. For each lane, five slices (250–150 kDa, 150–75 kDa, 75–50 kDa, 50–25 kDa, and 25–10 kDa) were excised and subjected to in-gel trypsin digestion, peptide purification and HPLC-ESI-MS/MS analysis using an LTQ Orbitrap Elite as described[11]. Peptide/protein identification and quantification were performed using the MaxQuant, one of the popular quantitative proteomics software packages [18]. Peak areas (PAs) for each protein were obtained by selecting the label-free quantification option in MaxQuant. Only proteins identified with minimum of 2 unique peptides were considered (Fig. 1B). To be considered as a PP2Ac interaction partner, a protein has to further satisfy the following two criteria:

1) Proteins have an enrichment ratio larger than 10, or only identified in the PP2Ac co-immunoprecipitates but not identified in any of the eight NIGG control samples. The calculation of enrichment ratio was described in our previous publication[18]. Briefly, PA for a protein identified in a gel lane was normalized against the sum of the peak areas for all proteins identified in the same gel lane to obtain normalized ratio for each protein, Norm: i ,

$$\text{Norm:}i = \frac{\text{PA}_i}{\sum_1^n \text{PA}_i}$$

Then, the average of normalized ratio for each protein in the PP2Ac co-immunoprecipitates, $Average_Norm:i_PP2Ac$, as well as the average of normalized ratio for the same protein in the NIGG co-immunoprecipitates, $Average_Norm:i_NIGG$, were obtained. Finally, $Average_Norm:i_PP2Ac$ was divided by $Average_Norm:i_NIGG$, to obtain the enrichment ratio for each protein.

$$Enrichment_Ratio:i = \frac{Average_Norm:i_PP2Ac}{Average_Norm:i_NIGG}$$

Since we used NIGG as a control, the first level of identification will be to search for proteins exclusively detected in the PP2Ac co-immunoprecipitates. However, a trace amount of a protein absorbed non-specifically on the NIGG beads may be identified with minimum of 2 unique peptides with false discovery rate (FDR) at 0.01 due to the high sensitivity of our approach, therefore resulting in false negatives. Nonetheless, if this protein is a true component of the PP2A complex, higher peak area will be assigned to this protein in the PP2Ac sample than in the NIGG sample. In this study, we used a cutoff of enrichment ratio larger than 10.

2) Proteins are identified with label-free quantification PAs in more than half (e.g., >4 out of 8) of the PP2Ac co-immunoprecipitates.

To determine the relative quantities of PP2Ac interaction partners in INS-1 832/13 β -cells under basal and glucotoxic conditions, the PA for each protein identified in a specific sample was normalized against the PA for PP2Ac identified in the same sample, which results in $Norm:j$.

$$Norm:j = \frac{PA_j}{PA_PP2Ac}$$

The normalization strategy is widely used in proteomics studies involving protein-protein interactions [19], and uses the same concept used in western blotting, in which the western blot signal for an interaction protein is normalized against that for the protein serving as the “bait.” The normalized peak area for each PP2Ac interaction partner, $Norm:j$, was compared between the low glucose and high glucose conditions to determine effects of glucotoxicity on protein-protein interactions involving PP2Ac.

To be considered as glucotoxic responsive PP2Ac interaction partners, a PP2Ac interaction partner protein has to further satisfy two criteria: 1) Has >1.5 fold change of normalized peak areas (basal vs. glucotoxic, $n = 4$); 2) Has significantly changed normalized peak areas ($P < 0.05$ assessed by independent Student's t -test).

2.4. Bioinformatics

Pathway analysis on glucose-responsive PP2Ac interaction partners were performed using Ingenuity Pathway Analysis (Ingenuity Systems, Inc., Redwood City, CA; www.ingenuity.com), a bioinformatics analysis software package[20] that contains

biological and chemical interactions and functional annotations created by manual curation of the scientific literature[21].

2.5. Experimental validation of PP2Ac interaction partner

Glucose treatment of INS-1 832/13 cells and co-IP experiments were carried out as described in Methods section 2.2. The beads eluates were resolved on SDS-PAGE, transferred onto nitrocellulose membranes, and analyzed by western blotting with sheep polyclonal PPP2R1B antibody (Abcam, Cat. No. ab28350) and mouse monoclonal PP2Ac antibody. The immune complexes were detected by chemiluminescence.

To evaluate the protein abundance of PP2Ac interaction partners in INS-1 832/13 cells under basal and glucotoxic conditions, the treated cells were lysed in RIPA buffer (50 mM Tris.Cl, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% SDS with 1 mM PMSF, 10 µg/mL leupeptin, and 10 µg/mL aprotinin). The protein extracts were clarified by centrifugation, and quantified by Bradford method. For each sample, 70 µg of total protein was analyzed by western blot with PPP2R1B, PP2Ac and β -actin antibodies.

The density of protein bands on the western blot images were measured using ImageJ 1.50b (NIH). Student's *t*-test was used in statistical analysis.

3. Results

3.1. Identification of PP2Ac interaction partners in INS-1 832/13 β -cells

We used INS-1 832/13 β -cell line throughout the studies. This cell line mimics rodent and human islets in releasing insulin in response to physiological glucose concentrations[22]. Furthermore, recent studies from our laboratory have established close similarities between INS-1 832/13 cells and normal rat islets for PP2A regulation (e.g., carboxyl methylation of PP2Ac and catalytic activation). In addition, we recently demonstrated hyperactivation of PP2A in INS-1 832/13 cells and normal rat islets under the duress of glucotoxic conditions[8, 15]. As depicted in Fig. 1A, cells were treated at basal or glucotoxic conditions (2.5 vs. 25 mM; 48 hrs). Lysate proteins were precleared with NIGG, followed by co-immunoprecipitation with PP2Ac antibody. The proteins eluted from both anti-PP2Ac and NIGG protein A beads were resolved on 1D-SDS-PAGE, and subjected to in-gel trypsin digestion. The resulting peptides were desalted and analyzed by HPLC-ESI-MS/MS. As shown in Fig. 1B, 1131 proteins were identified in total from PP2Ac co-immunoprecipitations with minimum of 2 unique peptides with FDR at 0.01. Among them, 606 proteins had an enrichment ratio larger than 10. Out of these 606 proteins, 514 were identified with peak area (PA) in more than half (e.g., >4 out of 8) PP2Ac co-immunoprecipitates, which were classified as PP2Ac interaction partners (Supplemental Table 1), which represents the largest PP2Ac interactome to date.

Through comparison with protein interactions retrieved from BioGRID3.2 database (<http://thebiogrid.org/>), 38 out of 514 proteins identified in the present study were previously reported as PP2Ac interaction partners (Table 1), while the remaining 476 proteins were novel PP2Ac interaction partners. The previously reported PP2Ac interaction partners include the α and β isoforms of PP2A 65 kDa regulatory subunit A (PPP2R1A and

PPP2R1B), the α and δ isoforms of PP2A 55 kDa regulatory subunit B (PPP2R2A and PPP2R2D), the α isoform of PP2A 72/130 kDa regulatory subunit B (PPP2R3A), and the γ isoform of PP2 56 kDa regulatory subunit B (PPP2R5C). Most of these 38 interaction partners were identified in human cells. We, therefore, verified the interaction networks of the α and β isoforms of the human PP2Ac (PPP2CA and PPP2CB) at String Database (<http://string-db.org>), around half of high confident interaction partners were also identified in our study (Fig. 2). These known partners proved the effectiveness of our proteomic approach, although most of them were first identified in rat β -cells. Note that proteins may interact with PP2Ac directly or indirectly through another protein that interacts with PP2Ac directly.

Among the 476 novel PP2Ac interaction partners, there were more than 15 different kinases, such as the isoform CRA_a of LIM motif-containing protein kinase 1 (LIMK1), dual-specificity mitogen-activated protein kinase kinase 2 (MAP2K2), mitogen-activated protein kinase 1 (MAPK1) and calcium/calmodulin-dependent protein kinase type 1 (CAMK1). Some protein phosphatases (or their regulatory/catalytic subunits) were also identified as novel PP2Ac interaction partners, such as protein phosphatase 1 regulatory subunit 12A (PPP1R12A), the α isoform of protein phosphatase 3 catalytic subunit (PPP3CA), serine/threonine-protein phosphatase 4 regulatory subunit 1 (PPP4R1), and serine/threonine-protein phosphatase 6 catalytic subunit (PPP6C). We also found that insulin-degrading enzyme (IDE), UDP-glucose:glycoprotein glucosyltransferase 1 (UGGT1) and voltage-dependent anion-selective channel protein 1 (VDAC1) as PP2Ac associated proteins. Additionally, more than 20 ribosomal proteins, numerous translation initiation factors as well as Ras-related proteins were also in the list of novel PP2Ac partners that we identified in the current study.

3.2. Glucose responsive PP2Ac interaction partners

As can be seen from Fig. 1B, 265 out of the 514 PP2Ac interaction partners, had a fold change greater than 1.5 (i.e., 1.5 fold increase) or less than 0.67 (i.e., 1.5 fold decrease) when comparing samples treated at basal and glucotoxic conditions. Among these, 89 proteins showed a significant difference in PP2Ac interaction in response to the glucotoxic treatment ($P < 0.05$), and they were considered as glucose responsive PP2Ac interaction partners (Supplemental Table 2). Among these, seven proteins have been reported to interact with PP2Ac in other cell models, such as PPP2R1B[19, 23–26] and PPP2R2A[19, 23, 27]. In response to high glucose treatment, the abundance of PPP2R1B and PPP2R2A interacting with PP2Ac in rat β -cells was increased to 1.83 and 2.32 folds, respectively. The other three PP2Ac partners, cortactin binding protein 2 (CTTNBP2)[24], sarcolemmal membrane-associated protein (SLMAP)[19] and Ints5 protein[28], also showed 11.03, 4.95 and 2.47 fold increased association with PP2Ac upon the high glucose treatment. In contrast, protein phosphatase 1B (PPM1B)[29], also previously reported as PP2Ac partner, exhibited a reduced interaction with PP2Ac (0.27 fold change or 3.7 fold decrease) in response to the high glucose treatment.

There were 13 PP2Ac interaction partners with a fold change greater than 5 or less than 0.2 (also with $P < 0.01$) in response to high glucose treatment (Table 2). Protein peripherin

showed decreased association with PP2Ac (0.19 fold change or 5.2 fold decrease). All other proteins had increased association with PP2Ac ranged from 5–23 fold increase. For example, LIM domain-containing protein 1 (LIMD1) and the isoform CRA_a of LIM motif-containing protein kinase 1 (LIMK1) increased 12.94 fold and 9.85 fold, respectively. The subunit 4 (predicted) isoform CRA_a of actin related protein 2/3 complex (ARPC4) showed a 7.08 fold increase in its association with PP2Ac upon the high glucose treatment.

3.3. Glucose responsive PP2Ac interaction partners related to insulin secretion

Ingenuity Pathway Analysis of the 514 PP2Ac interaction partners revealed 59 significantly enriched pathways ($P < 0.01$ and with minimum of four interaction partners in a specific pathway) (Supplemental Table 3). Most of the pathways are related to protein synthesis and degradation, cytoskeleton dynamics, as well as AMPK signalling. On the other side, much less glucose responsive PP2Ac partners were identified in these pathways, and limited to few proteins including PPP2R2A, PPP2R1B, RhoA, LIMK1 and ARPC4. Pathways with minimum of three glucose responsive PP2Ac partners were shown in Fig. 3. The insulin secretion pathway was not enriched through the PP2Ac immunoprecipitation by the IPA analysis. Albeit, manual literature search revealed a number of proteins involved in insulin secretion and other related cell functions (Fig. 4). As shown in the figure, APPL1, RHOA, RAB10, PLA2G6, PFKFB2 and EIF2C2 have been shown to regulate insulin secretion. Protein PPP4R1 and CIAPIN1 are involved in anti-apoptosis of β -cells, thus retaining islet survival and function, including insulin secretion. We also found a number of proteins related to vesicle trafficking (VPS52, VPS37A, TSG101, RAB5C, RAB10 and EEA1). All of the five identified ribosomal components (RPL9, RPL4, RPL30, RPL18A and MRPL35) were found with increased association with PP2Ac in response to glucotoxic treatment. Nine proteins (VGLL4, TRIP11, STAT6, PHF5A, NCOR1, LIMD1, ILF3, HIST1H1C and DDX17) involved in transcription regulation were also identified.

3.4. Experimental validation of PPP2R1B as a glucose responsive PP2Ac interaction partners

PPP2R1B is the β isoform of PP2A scaffolding A subunit, and was identified as a glucose responsive PP2Ac interaction partners by mass spectrometry in this study (Supplemental Table 2). As an example, we validated this finding by co-IP and western blot using proteins from INS-1 832/13 cells which were treated at basal (2.5 mM) or glucotoxic (25 mM) conditions. As shown in Fig. 5A, association of PPP2R1B with PP2Ac was significantly higher (1.57 fold) under glucotoxic condition compared to basal glucose conditions ($n = 4$, $P < 0.05$). This is consistent with our proteomics findings indicating that the abundance of PPP2R1B interacting with PP2Ac in rat β -cells was increased to 1.83 folds in response to high glucose treatment ($n = 4$, $P < 0.05$). In addition, we quantified PPP2R1B abundance in INS-1 832/13 cells. As shown in Fig. 5B, although the PPP2R1B abundance at glucotoxic condition was only 1.13 fold over the basal one when normalized to β -actin level ($P > 0.05$), glucotoxic treatment significantly increased the ratio of PPP2R1B/PP2Ac (1.47 folds vs. low glucose treatment, $n = 4$, $P < 0.01$).

4. Discussion

The PP2A family of enzymes has been implicated in the regulation of a variety of cellular functions including hormone secretion, growth, survival and apoptosis[4, 6, 7]. PP2A accounts for ~1% of total cellular protein and ~ 80% of total serine/threonine phosphatases activity[8], thus representing a major class of protein phosphatases in mammalian cells. Despite significant advances in our current understanding of regulation of cellular function by PP2A under physiological conditions, little is understood with regard to its regulation under various pathological conditions, such as diabetes. Emerging evidence suggests hyperactivation of PP2A in liver, muscle, retina and the pancreatic islet under the duress of glucolipotoxicity and diabetes. Interestingly, pharmacological inhibition of PP2A or siRNA-mediated depletion of PP2Ac levels largely restored PP2A activity to near normal levels under these conditions[15]. One of the principal objectives of the current study was to identify new protein interaction partners of PP2Ac, using a novel proteomics approach, in insulin-secreting INS-1 832/13 cells following 48 hrs exposure to basal (2.5 mM) or glucotoxic (25 mM) conditions. Salient features of our study are: [i] identification of 514 protein interaction partners, out of which 476 are novel and [ii] identification of at least 89 proteins whose interaction was significantly increased with PP2Ac under glucotoxic condition. A detailed literature search revealed a number of interaction partners identified in our study play essential roles in islet function including insulin secretion as discussed below.

4.1. PP2Ac interaction with various signaling proteins necessary for physiological insulin secretion

As depicted in Figure 4, we identified several interaction proteins of PP2Ac, which have been implicated in islet function and insulin secretion. They include a variety of small molecular mass G proteins (Rac1, Rho A, Rab5c) and proteins involved in protein sorting and trafficking (GGA2 and SRP72) and vesicle trafficking (VPS52, VPS37A, Rab10, Rab5C etc.). These findings suggest a close interaction between these signaling proteins and PP2Ac. In this context, several studies from our laboratory and others have implicated requisite roles of small G-proteins (Rac1, Cdc42, Rho A and Rab) in GSIS[30, 31]. These signaling proteins are also important for vesicular trafficking and cytoskeletal remodeling to enable fusion of insulin secretory granules with the plasma membrane for insulin secretion[30, 31].

In the context of actin cytoskeletal remodeling, our current findings also suggest significant cross-talk between PP2Ac and LIMK1. It is well documented that LIMK1, a serine/threonine-protein kinase, plays an essential role in the regulation of actin filament dynamics. It acts downstream of several Rho family GTPase signal transduction pathways. Activation of upstream kinases including ROCK1, PAK1 and PAK4 leads to phosphorylation and activation of LIMK1, which subsequently phosphorylates and inactivates the actin binding/depolymerizing factors[32, 33] culminating in the prevention of cleavage of F-actin and stabilization of actin cytoskeleton. Furthermore, LIMK1 has been shown to regulate several actin-dependent biological processes including cell motility, cell cycle progression, and differentiation[34]. Together, our findings provide the first evidence for interaction between

PP2Ac and key signaling proteins involved in protein/vesicle trafficking and acting cytoskeletal remodeling, which are essential for GSIS to occur.

We also identified immunoglobulin-binding proteins (Igbp1) as one of the interaction partners of PP2Ac (Supplemental Table 1). Igbp1 is also referred to as $\alpha 4$ (or Tap42 in yeast), which is a non-canonical adaptor subunit of PP2A[35]. In addition to forming complexes with the A and B subunits, PP2Ac has been shown to interact with other adaptor regulatory subunits, including $\alpha 4$. Recent evidence supports novel roles for $\alpha 4$ in PP2A biogenesis, stability and activation[35, 36]. It forms a complex with PP2Ac; such an interaction retains PP2Ac in its catalytically inactive conformation, which is resistant to ubiquitination and proteasomal degradation[37]. Interestingly, besides PP2A, $\alpha 4$ has been shown to interact with other phosphatases including protein phosphatase 4 (PP4) and protein phosphatase 4 (PP6)[36]. Our current findings also identified PP4 as one of the interacting partners of PP2Ac. In this context, we provided the first evidence for nuclear localization and regulation of PP4 in insulin-secreting cells, and reported its involvement in the induction of defects in nuclear lamin processing induced by cytokines. A potential link between $\alpha 4$ and CML of PP2Ac has been suggested, but not verified conclusively. Recent data from our laboratory suggested expression of $\alpha 4$ in INS-1 832/13 cells and normal rodent islets. Additional studies are needed to further verify roles of $\alpha 4$ in the survival and apoptosis of the islet β -cell.

4.2. PP2Ac interaction with key signaling proteins known to regulate cell dysfunction and apoptosis

Data from our current investigation indicate potential interaction between PP2Ac and protein methyl esterase-1 (PME-1). Original investigations from our laboratory have suggested that PP2Ac undergoes methylation-demethylation at the C-terminal leucine (Leu-309) residue. We have also characterized both methylating and demethylating in pancreatic β -cells[38]. It appears that C-terminal methylation of PP2Ac is increased by glucose under acute as well as chronic exposure conditions, thus controlling GSIS and cell apoptosis[15, 38]. In support of this, we have recently demonstrated that siRNA-mediated knockdown of LCMT-1 significantly attenuated the carboxymethylation of PP2Ac and hyperactivation of PP2A under glucotoxic conditions, suggesting that the carboxymethylation causes sustained activation of the enzyme[8, 15]. Despite this evidence potential regulatory roles of PME-1 in islet function remain unexplored. Together, our current findings suggest a close interaction between PP2Ac and LCMT-1 and PME-1, the methylating and demethylating enzymes, respectively.

Data from our current studies suggested a significant increase (~3.6 fold) in the interaction between PP2Ac and PPP4R1, which is a regulatory subunit of PP4. In this context, previous studies from our laboratory have demonstrated a high level of expression of catalytic subunit of protein phosphatase 4 (PP4c) in the nuclear fraction isolated from pancreatic β -cells[39]. Moreover, exposure of islet β -cells to IL-1 β , a proinflammatory cytokine, resulted in a marked increase in NO release with concomitant reduction in the degree of expression and the carboxymethylation of PP4C in the nuclear fraction. Immunoprecipitation studies suggested potential complexation of PP4c with nuclear lamin-B, a key regulatory protein involved in the nuclear envelope assembly. Based on these findings, we proposed that

IL-1 β -mediated inhibition of PP4 activity might result in the retention of lamin-B in its phosphorylated state, which is a requisite for its degradation by caspases leading to the apoptotic demise of the β -cell[39]. Potential regulatory roles of PP4 in islet β -cell dysfunction induced by glucotoxic conditions remain to be determined.

We also noted a significant increase (2.3 folds) in the interaction between PP2Ac and B55 α in cells exposed to glucotoxic conditions. Several recent studies implicated this regulatory subunit in the onset of metabolic dysfunction of the islet β -cell. For example, recent studies by Yan et al have demonstrated involvement of a B55 α -containing PP2A holoenzyme in the dephosphorylation of FOXO1 in islet β -cells under conditions of H₂O₂-induced oxidative stress[40]. These investigators have also reported increased expression of B55 α subunits in islets derived from *db/db* mouse[40]. In addition, we recently reported a significant increase (1.5 fold) in the expression of B55 α subunit in INS-1 832/13 cells exposed to hyperglycemic conditions[15]. It is likely that such pathological conditions promote the holoenzyme assembly of PP2A by increasing its carboxylmethylation, which, in turn, promotes interaction between the regulatory, structural and catalytic subunits culminating in the catalytic activation of the enzyme[8, 15].

5. Conclusions

We performed the first global analysis of PP2Ac interaction partners in pancreatic islet β -cells under basal and glucotoxic conditions. In total, 514 proteins were identified as PP2Ac interaction partners. Among them, 476 are novel, and 481 were exclusively identified in the PP2Ac co-IP, but not in any of the 8 NiG immunoprecipitates at all. This represents the identification of the largest PP2Ac interactome to date. Moreover, western blotting analysis validated PPP2R1B as a glucose responsive PP2Ac interaction partners. Furthermore, we provided the first global view of PP2Ac protein-protein interactions caused by high glucose treatment, and discovered numerous PP2Ac interaction partners in multiple pathways in the context of glucotoxicity and islet dysregulation. It is hoped that this information will facilitate the design of experiments to better understand the molecular mechanism responsible for metabolic dysfunction and demise of pancreatic β -cells under glucotoxic conditions leading to the loss of functional β -cell mass.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

1D-SDS-PAGE	One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis
co-IP	co-immunoprecipitation
FDR	false discovery rate
GSIS	glucose-stimulated insulin secretion
HG	high glucose
INS-1 832/13	a glucose-responsive insulin-secreting pancreatic β -cell line
IRS1	insulin receptor substrate-1
LIMK1	LIM motif-containing protein kinase 1
NIgG	normal mouse IgG
PA	peak area
PP2A	serine/threonine protein phosphatase 2A
PP2Ac	PP2A catalytic subunit
PP4	protein phosphatase 4
PPP2CA	α isoform of PP2Ac
PPP2CB	β isoform of PP2Ac
PPP2R1B	β isoforms of PP2A 65 kDa regulatory subunit A

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Highlights

- Identified the largest PP2Ac interactome to date.
- Provided the first global view of PP2Ac interaction under hyperglycemic condition.
- PP2A is involved in multiple signaling pathways in β cells.

Fig. 1.

Flowchart of experimental procedure. A. Experimental design of the study. β -cells were treated at basal (2.5 mM) or glucotoxic (25 mM) levels of glucose. Proteins associated with PP2Ac were co-immunoprecipitated and identified by mass spectrometry. Proteins bound to NlgG were not considered P2Ac interaction partners unless their enrichment ratio were larger than 10. PP2Ac partners responsive to high glucose treatment were further identified and used in bioinformatics analysis. * Proteins were eluted from the beads, and analyzed by HPLC-ESI-MS/MS as described in Methods section 2.3. B. Stepwise identification of glucose responsive PP2Ac interaction partners. Criteria were given at each step, and the number of proteins meet the criteria was shown in the parenthesis.

Fig. 2. Protein interaction networks. From <http://string-db.org>, twenty interactors with confidence larger than 0.99 were employed to compose the interaction network of PPP2CA (A) and PPP2CB (B). Proteins identified in this study are highlighted in red circles.

Fig. 3.

Pathway analysis of glucose responsive PP2Ac interaction partners. Only pathway containing minimum of three identified glucose-responsive PP2Ac partners are shown. The purple and blue bars indicate the numbers of glucose responsive and nonresponsive PP2Ac partners, respectively.

Fig. 4. Summary of glucose-responsive PP2Ac interaction partners. In response to high glucose treatment, the proteins with increased PP2Ac association are highlighted in red, and the ones with decreased association are highlighted in green. Please note that some proteins may be involved in function categories.

Fig. 5.

Experimental validation of PPP2R1B as a glucose responsive PP2Ac interaction partner. A. Co-IP of PPP2R1B with PP2Ac. INS-1 832/13 cells were exposed to basal (2.5 mM) or glucotoxic (25 mM) conditions, and total protein (lysate) was extracted, and incubated with PP2Ac specific antibody. Co-immunoprecipitated PPP2R1B was visualized by western blot analysis. As control, proteins bound to NIgG were analyzed by western blot using PPP2R1B and PP2Ac specific antibodies. Quantification of the proteins is shown in the graph (n = 4). Bars represent standard deviation. B. Measurement of PPP2R1B abundance. PPP2R1B abundance in INS-1 832/13 cells at basal and glucotoxic conditions were evaluated using whole cell lysate. Quantification of the proteins is shown in the graphs (n = 4). Bars represent standard deviation.

Table 1

Thirty-eight reported PP2Ac interaction partners were identified in pancreatic β -cells

Gene name	Protein ID	Protein name	MW [kDa]	Enrichment ratio	Reference	Species*
Ankle2	Q7TP65	Ankyrin repeat and LEM domain-containing protein 2	106.4	1000 [#]	[19]	H
Arpe4	B2RZ72	Actin related protein 2/3 complex, subunit 4 (Predicted), isoform CRA_a	19.7	1000	[41]	H
Cdk2	D3ZJC8	Cyclin-dependent kinase 2	39.0	1000	[29, 42]	H
Csnk1a1	D3ZRE3	Casein kinase I isoform α	41.9	1000	[43]	H
Ctnbp2	Q2IBD4	Cortactin binding protein 2	178.8	1000	[24]	H
Ctnbp2nl	D4A8-8	CTTNBP2 N-terminal like (Predicted), isoform CRA_a	70.1	1000	[24]	H
Fam40a	G3V8E2	Protein Fam40a	95.6	1000	[24]	H
Fgfr1op	Q4V7C1	FGFR1 oncogene partner	43.0	1000	[24, 44]	H, M
Fgfr1op2	Q6TA25	FGFR1 oncogene partner 2 homolog	29.4	1000	[19]	H
Gga1	F1L PF4	Protein Gga1	70.0	1000	[45]	H
Hmnpa2b1	A7VJC2	Heterogeneous nuclear ribonucleoproteins A2/B1	37.5	1000	[46]	M
Igbp1	O08836	Immunoglobulin-binding protein 1	39.1	1000	[19, 24, 37, 47-55]	H, M, R
Ints3	D3ZUT9	Protein Ints3	117.9	1000	[28]	H
Ints5	D3ZTW1	Protein Ints5	108.4	1000	[28]	H
Map3k7ip1	D4A6C6	Protein Map3k7ip1	54.6	1000	[48]	H
Mapk3	P21708-2	Isoform 2 of mitogen-activated protein kinase 3	45.8	1000	[56]	H
Mob4	Q9QYW3	MOB-like protein phocein	26.0	1000	[19, 24]	H
Myh10	F1L Q02	Myosin-10	233.6	1000	[19]	H
Pcdl10	Q6NX65	Programmed cell death protein 10	24.4	1000	[24]	H
Polr1	F1L RJ6	DNA polymerase	166.9	1000	[57]	H
Ppf1a1	D3ZXH0	Protein Ppf1a1	142.7	1000	[19, 24]	H
Ppm1b	Q99ND8	Ppm1b protein	51.0	1000	[29]	H
Ppme1	Q4FZT2	Protein phosphatase methyltransferase 1	42.3	1000	[19, 24]	H
Ppp2r1a	Q5X134	PP2 65 kDa regulatory subunit A, α isoform	65.3	176.7	[19, 23-27, 36, 41, 44, 58-63]	H, M
Ppp2r1b	D4A1Y3	PP2 65 kDa regulatory subunit A, β isoform	76.1	1000	[19, 23-26]	H
Ppp2r2a	P36876	PP2 55 kDa regulatory subunit B, α isoform	51.7	1000	[19, 23, 24, 27, 64]	H, M

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Gene name	Protein ID	Protein name	MW [kDa]	Enrichment ratio	Reference	Species*
Ppp2r2d	P56932	PP2 55 kDa regulatory subunit B, δ isoform	52.0	1000	[19, 24, 65]	H
Ppp2r3a	D3ZLD7	PP2 72/130 kDa regulatory subunit B, α isoform	129.9	1000	[66, 67]	H, M
Ppp4c	G3V8M5	PP4 catalytic subunit	35.1	1000	[68]	H
Ppp2r5c	D4A1A5	PP2 56 kDa regulatory subunit B, γ isoform	59.9	1000	[19, 24, 69–72]	H
Prkaa1	P54645	5-AMP-activated protein kinase catalytic subunit α -1	64.0	1000	[73, 74]	H
Rplp1	P19944	60S acidic ribosomal protein P1	11.5	1000	[75]	H
Sike1	Q5FWT9	Suppressor of IKBKE 1	23.6	1000	[19]	H
Slmap	F1LM85	Sarcolemmal membrane-associated protein	98.2	1000	[19]	H
Strn	G3V6L8	RCG61894, isoform CRA_a	86.1	1000	[19, 24]	H
Strn4	F1M6V8	Protein Strn4	81.4	1000	[19, 24]	H
Ttpo3	D4AAM0	Protein Ttpo3	104.9	1000	[41]	H
Uba52	P62986	Ubiquitin-60S ribosomal protein L40	14.7	1000	[49]	H

* H: human; M: mouse; R: rat.

only identified in PP2Ac IP.

Table 2

Glucose responsive PP2Ac partners with fold change > 5 and $P < 0.01$.

Gene name	Protein ID	Protein name	MW [kDa]	P value	Fold change
Arpe4	B2RZ72	Actin related protein 2/3 complex, subunit 4 (Predicted), isoform CRA_a	19.7	0.0048	7.1
Ciapiin1	Q5XID1	Anamorsin	33.0	0.0035	5.0
Ctnbp2	Q2IBD4	Cortactin binding protein 2	178.8	0.0002	11.0
Hmmr	D4A2M9	Hyaluronan mediated motility receptor (RHAMM)	82.9	0.0036	12.6
Limd1	B5DEH0	LIM domain-containing protein 1	71.4	0.0017	12.9
Limk1	G3V663	LIM motif-containing protein kinase 1, isoform CRA_a	72.6	0.0010	9.8
Micall2	D3ZEN0	Protein Micall2	107.8	0.0043	6.6
Mrpl35	D3ZE10	Mitochondrial ribosomal protein L35 (Predicted), isoform CRA_a	21.5	0.0065	13.1
Ncor1	FILSA0	Nuclear receptor corepressor 1	271.2	0.0074	7.6
Prph	P21807	Peripherin	53.5	0.0000	0.2
Pold1	G3V8M1	DNA polymerase	123.6	0.0016	5.3
Txnrd1	O89049	Thioredoxin reductase 1, cytoplasmic	54.7	0.0008	23.3
Vgll4	Q5BJP0	Protein Vgll4	31.0	0.0066	8.2