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Glucose regulates protein kinase CK2 in pancreatic β-cells and its interaction with PDX-1

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ABSTRACT

The pancreatic duodenal homeodomain transcription factor PDX-1 plays a pivotal role in the development of the pancreas and the maintenance of glucose homeostasis by pancreatic β-cells. Recently, we found that the highly conserved, ubiquitously expressed tetrameric Ser/Thr protein kinase CK2, which is formed by two catalytic subunits (α and/or α′) and two non-catalytic subunits (β), phosphorylates PDX-1. So far, only little is known about CK2 in pancreatic β-cells and how this enzyme is regulated in these cells. In the present study, we found that (i) CK2 binds to PDX-1, (ii) the binding between CK2 and PDX-1 is regulated by glucose, (iii) glucose modulates the subcellular localization of PDX-1 and CK2 and (iv) the kinase activity is also regulated by glucose.

Our novel data indicate that CK2 is a co-factor of PDX-1 in response to glucose in pancreatic β-cells.
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1. Introduction

Diabetes mellitus type 2 is a common metabolic disorder, which is caused by insulin resistance or by pancreatic β-cell dysfunction. Pancreatic β-cells maintain blood glucose homeostasis by the production and secretion of insulin upon glucose stimulation. One of the key regulators of insulin production is the transcription factor PDX-1 (Ohlsson et al., 1993). The PDX-1 gene product is expressed in early pancreatic endocrine, exocrine and ductal progenitor cells (Jonsson et al., 1995; Offield et al., 1996; Ahlgren et al., 1998). Under diabetic conditions, the expression and/or activation of PDX-1 is reduced. PDX-1 knock-out mice express a severe diabetic phenotype due to β-cell dysfunction (Gannon et al., 2008). PDX-1, also known as IPF1 (Ohlsson et al., 1993), IDX-1 (Miller et al., 1994), STF1 (Leonard et al., 1993), or IUF1 (Boam and Docherty, 1989) is a homeodomain transcription factor which, in addition to the insulin promoter, also binds to and regulates the glut 2 promoter (Waeber et al., 1996) and the glucokinase promoter (Watada et al., 1996). Regulation of gene expression is achieved by binding to other transcription factors, co-activators and co-repressors (for review see: Al-Quobaili and Montenarh, 2008). Transcriptional activity of PDX-1 is also regulated by post translational modifications such as phosphorylation (Boucher et al., 2006; Lebrun et al., 2005; Meng et al., 2010a; An et al., 2006, 2010; Elrick and Docherty, 2001; Humphrey et al., 2010), sumoylation (Kishi et al., 2003) or glycosylation (Gao et al., 2003). The PDX-1 protein sequence and domain structure is highly conserved among different species. The N-terminus contains the transactivation domain. The middle region contains the homeodomain, which is responsible for DNA binding and protein-protein interactions. The C-terminus harbours the interaction domain with various co-factors and some of the various phosphorylation sites (for review see: Al-Quobaili and Montenarh, 2008). Within the homeobox domain, PDX-1 contains an Antennapedia-like domain which facilitates penetration into cells (Noguchi et al., 2003) and a nuclear localization signal (NLS) (Moede et al., 1999; Hessabi et al., 1999).

Several studies have shown that glucose stimulated phosphorylation of PDX-1 regulates its DNA binding activity (Petersen et al., 1998; Wu et al., 1999) and the cytoplasmic nuclear shuttling (Macfarlane et al., 1999). Another study has reported that glucose stimulation of primary islets or pancreatic β-cells decreases PDX-1 phosphorylation (Humphrey et al., 2010). It has been shown that PDX-1 exists in the cytoplasm of islets that were incubated in low glucose. In contrast, Molde et al. reported that there is no glucose-dependent cytoplasmic-nuclear cycling of PDX-1 (Moede et al., 1999). Yet another study has demonstrated that in unstimulated

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cells, PDX-1 is associated with the nuclear periphery with some staining in the cytoplasm. Upon stimulation with glucose, PDX-1 is rapidly translocated into the nucleoplasm (Elrick and Docherty, 2001). So far, it is very difficult to explain these diverse observations.

We recently found that PDX-1 is phosphorylated by protein kinase CK2 (Meng et al., 2010a). This PDX-1 phosphorylation is implicated in the regulation of insulin production and release from pancreatic β-cells (Meng et al., 2010a,b). Protein kinase CK2 is a pleiotropic enzyme, which is highly conserved in the animal kingdom (Pinna, 1997). Thornburg and Lindell first described CK2 as a multi-subunit protein kinase where the holoenzyme is generated by the association of two α- or α′-subunits with a dimer of the β-subunit (Thornburg and Lindell, 1977). Loss of either CK2α or CK2β is lethal (Lou et al., 2007; Buchou et al., 2003). So far nothing is known about the regulation of CK2 in pancreatic β-cells.

In the present study, we therefore analyzed the interaction between CK2 and PDX-1 in pancreatic β-cells under low and high glucose conditions by co-localization experiments and co-immunoprecipitation. Furthermore, we analyzed the cytoplasmic/nuclear translocation of CK2 and PDX-1 under various glucose concentrations. Additionally, CK2 kinase activity as well as its binding to PDX-1 was analyzed depending on glucose concentrations. Our data show for the first time a glucose dependent regulation of (i) the enzymatic activity of CK2, (ii) its interaction with PDX-1 and (iii) the subcellular localization of PDX-1 and CK2. These results strongly suggest CK2 as a co-factor in the response of pancreatic β-cells to variations in the glucose level.

2. Methods

2.1. Cell culture and antibodies

The mouse insulinoma cell line MIN6 (Ishihara et al., 1993) was maintained according to (Meng et al., 2010a,b). This cell line behaves like primary β-cells in response to variations in glucose concentration. Detection of CK2 was performed by using the mouse monoclonal antibody 1A5 (Schuster et al., 2001) or the rabbit antibody #26 against CK2α (Faust et al., 1999), the rabbit antibody #30 against CK2α (Faust et al., 1999) and the mouse monoclonal antibody 6D5 (Nastainczyk et al., 1995) or the rabbit antibody #32 against CK2β (Faust et al., 1999). PDX-1 was identified with a polyclonal rabbit antiserum against recombinant whole length mouse PDX-1 as well as the mouse monoclonal antibody PDX-1 Clone 267712 (R&D Systems, Wiesbaden, Germany). For immunohistochemical staining, a guinea pig antibody against PDX-1 (Abcam, Cambridge, UK) was used. For the identification of the pancreatic cell types, we used mouse antibodies against insulin and glucagon as well as a rabbit antibody against somatostatin (all purchased from Abcam). For the detection of α-tubulin as a loading control, the mouse monoclonal antibody Clone DM1A (Sigma–Aldrich, Munich, Germany) was used.

2.2. Cell extraction, Western blot analysis and CK2 protein kinase assay

Cell extraction, Western blot and CK2 protein kinase assays were performed as described (Meng et al., 2010a). For protein detection, the following dilutions were used: polyclonal rabbit antibody (1:1000) against PDX-1 or mouse monoclonal PDX-1 antibody (1:500), mouse monoclonal antibody 1A5 (1:200) against CK2α, mouse monoclonal antibody 6D5 (1:50) against CK2β and rabbit antibody #30 against CK2α′ (1:1000) and mouse monoclonal antibody Clone DM1A for the detection of α-tubulin as a loading control (1:1000).

2.3. Protein extraction of primary mouse pancreas

Primary mouse pancreata were isolated from C57BL/6 mice (28–30 g body weight; Charles River, Sulzfeld, Germany). The animals were housed at the Institute for Clinical and Experimental Surgery, Saarland University, Germany, and had free access to tap water and standard pellet food (Altromin, Lage, Germany) until the experiments. All experiments were approved by the local governmental animal care committee and were conducted in accordance with the German legislation on protection of animals and the NIH Guidelines for the Care and Use of Laboratory Animals (NIH Publication #85-23, Rev. 1985). For protein extraction of primary mouse pancreas, the tissue sample frozen in liquid nitrogen was crushed in a mortar and then suspended in RIPA buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.5% Na-desoxycholate, 1% TritonX-100, 0.1% SDS containing the protease inhibitor cocktail Complete™, Roche Diagnostics, Mannheim Germany) and incubated for 1 h at 4 °C. The cell extract was centrifuged at 13,000 × *g* for 10 min. The supernatant was used for the determination of the protein content.

2.4. Co-immunoprecipitation

For co-immunoprecipitation experiments, MIN6 cells were incubated in DMEM with 5 mM and 25 mM glucose for 24 h. Cells were extracted and 4 mg of total protein in the cell extracts was subjected to immunoprecipitation. The cell lysates were pre-cleared twice with a mixture of protein A sepharose beads and CL 4-B sepharose beads (GE Healthcare, Freiburg, Germany) over a period of 1 h. The supernatant was incubated with a rabbit anti PDX-1 antibody for 2 h. Beads were washed four times with PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4). Bound proteins were eluted with SDS sample buffer (130 mM Tris–HCl, pH 6.8, 0.02% bromophenol blue (w/v), 10% β-mercaptoethanol, 20% glycerol (v/v), and 4% SDS) and analyzed by Western blot with the mouse monoclonal antibody 1A5 (1:200) against CK2α and the mouse monoclonal antibody 6D5 (1:50) against CK2β. Proteins were visualized by the ECL Lumilight system of Roche Diagnostics (Mannheim, Germany).

2.5. Immunofluorescence analysis

Immunofluorescence analysis was performed as described (Faust et al., 1999). For the identification of proteins, the mouse monoclonal antibody clone 267712 (1:50) against PDX-1 and the rabbit antibodies against CK2α (1:100) and CK2β (1:100) were used.

2.6. Immunohistochemical staining of primary mouse islets

Frozen sections of mouse pancreas including islets were fixed in acetone for 5 s and dried for 1 h at room temperature. After the sections were fixed with formaldehyde for 5 min at room temperature, they were washed in *aqua dest* followed by PBS, pH 7.4. For blocking of unspecific bindings, the sections were incubated with 3% goat serum in PBS, pH 7.4, for 30 min. The sections were then incubated with the primary antibodies as a mixture overnight in a humidified chamber at room temperature. PDX-1 was detected with the polyclonal rabbit antiserum (1:100) as well as with a guinea pig antibody (1:200). For the identification of the pancreatic cell type, mouse antibodies against insulin (1:500) or glucagon (1:1000) as well as a rabbit antibody against somatostatin (1:500) were used. After the sections were washed with PBS, pH 7.4, the secondary antibodies were incubated one after the other for 60 min at room temperature in a humidified chamber. As secondary antibody, goat anti rabbit Cy3 (Dianova, Hamburg, Germany, 1:400) and goat anti-guinea pig ALEXA-Fluor™ 488 (Invitrogen, Karlsruhe,

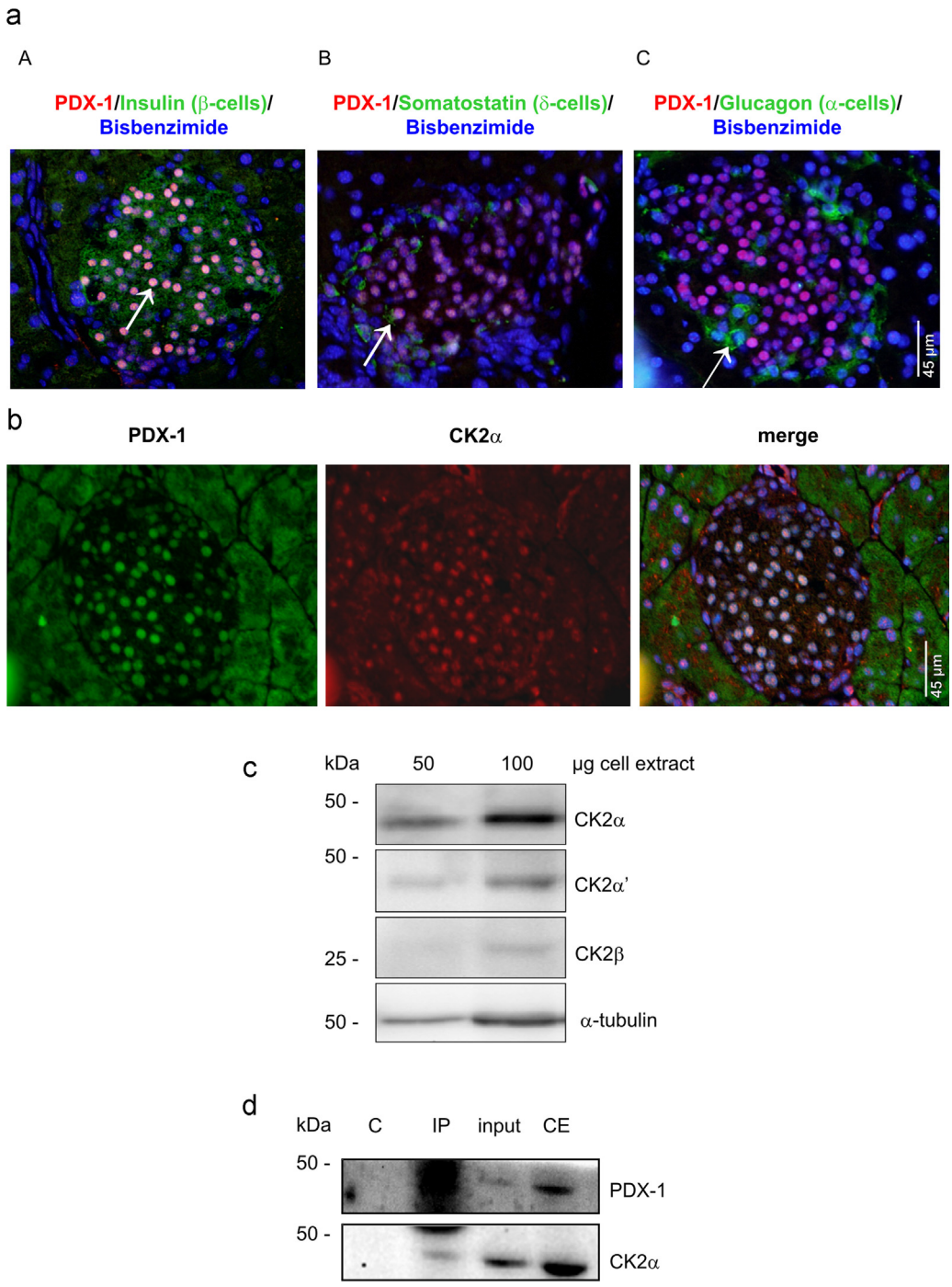

Fig. 1. Localization of PDX-1 and CK2 in primary mouse islets. (a) Co-localization of PDX-1 with insulin-, somatostatin- and glucagon-positive cells. Frozen sections of adult mouse pancreas were subjected to triple immunohistochemical staining using PDX-1 (guinea pig antibody for double staining of PDX-1 with somatostatin and glucagon, polyclonal rabbit antibody for double staining of PDX-1 with insulin) and anti-insulin/anti-somatostatin/anti-glucagon antibodies, as well as bisbenzimide. Goat-anti-rabbit-Cy3 and goat-anti-mouse-ALEXA-Fluor™ 488 were applied as secondary antibodies. Co-localization of PDX-1 with (A) insulin, (B) somatostatin, (C) glucagon. Arrows show A: insulin-positive cell (β-cell), B: somatostatin-positive cell (δ cell), C: glucagon-positive cell (α-cell) of the pancreatic mouse islet. (b) Co-localization of PDX-1 and CK2α in primary mouse islets. Frozen sections of adult mouse pancreas were subjected to double immunohistochemical staining using a guinea pig antibody against PDX-1 and CK2α mouse antibodies (1A5) as well as bisbenzimide. Goat-anti-rabbit-Cy3 and goat-anti-guinea pig ALEXA-Fluor™ 488 were applied as secondary antibodies. (c) Detection of CK2 subunits in cell extract of primary mouse pancreas. 50 µg or 100 µg of proteins in a total cell extract of primary mouse pancreas was analyzed on a 12.5% polyacrylamide gel followed by Western blot with mouse monoclonal antibody 1A5 against CK2α, mouse monoclonal antibody 6D5 against CK2β, rabbit antibody #30 against CK2α′ and mouse monoclonal antibody clone DM1A against α-tubulin as a loading control. Protein bands were visualized by the ECL Lumilight system of Roche Diagnostics (Mannheim, Germany). (d) Co-immunoprecipitation of PDX-1 and CK2α in cell extract of primary mouse pancreas. A cell lysate from mouse pancreatic cells was precleared twice with a mixture of protein A sepharose beads and CL 4-B agarose beads. The supernatants were incubated with the polyclonal rabbit PDX-1 antibody. The immunoprecipitated proteins were separated by 10% polyacrylamide gel electrophoresis, transferred to a PVDF membrane and analyzed by Western blot with the polyclonal rabbit antibody against PDX-1 and the mouse monoclonal antibody 1A5 against CK2α. Protein bands were visualized by the ECL Lumilight system of Roche Diagnostics (Mannheim Germany) input: 10% of cell extract was used in the experiment, CE: cell extract (100 µg), C: precipitate with protein A sepharose alone as control, IP: immunoprecipitate.

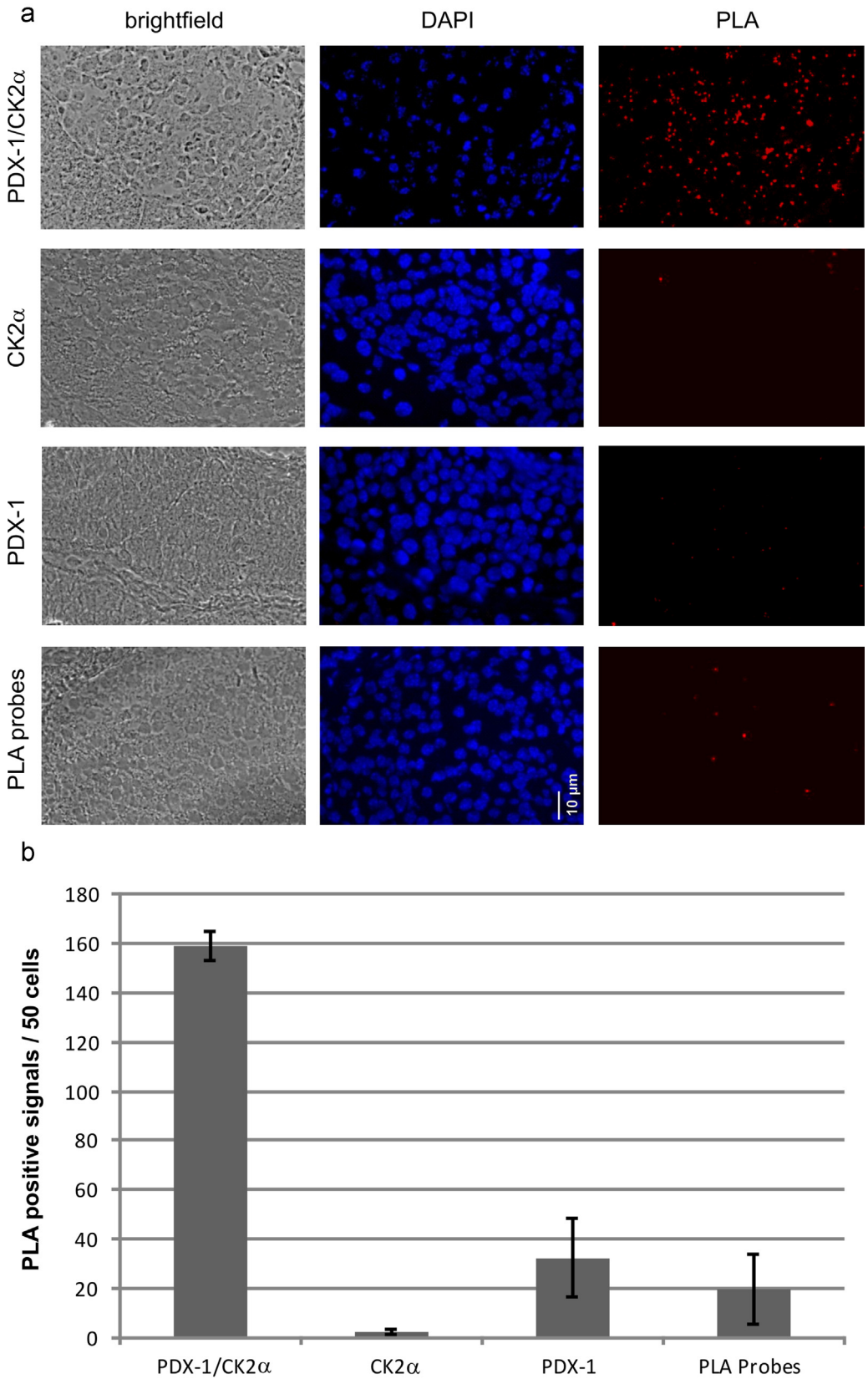

Fig. 2. Duolink® *in situ* Proximity Ligation Assay (PLA) of PDX-1 and CK2α in mouse islets. (a) The frozen sections of adult mouse pancreas were subjected to Duolink® *in situ* Proximity Ligation Assay according to manufacturer's instructions using a double staining of the islets with polyclonal rabbit antibodies against PDX-1 and mouse monoclonal antibody 1A5 against CK2α. For negative control, a single staining with the PDX-1 and CK2α antibodies as well as the PLA probes was performed. (b) Single dots were counted in 50 cells of three different areas (total number of cells 150).

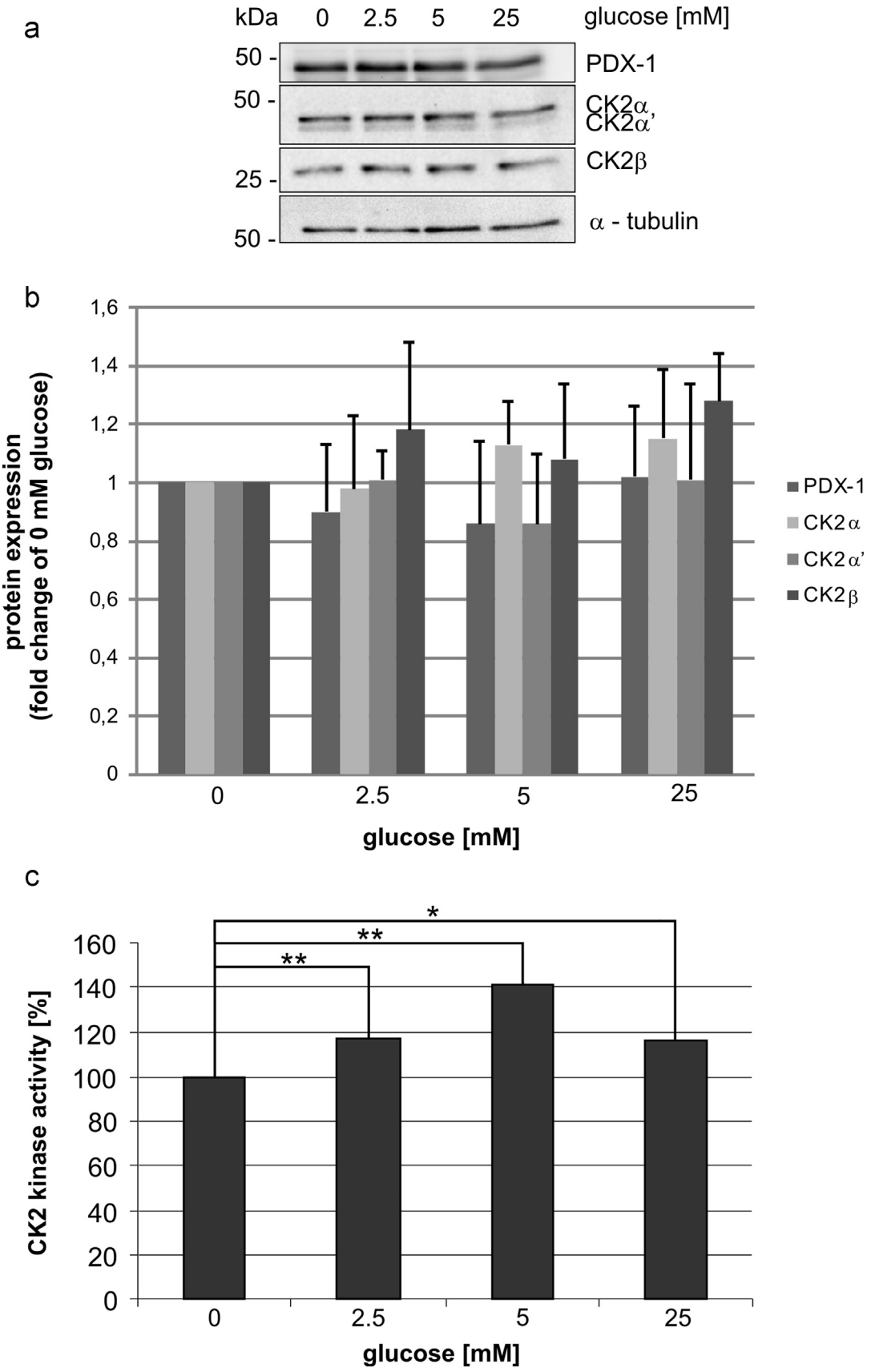

Fig. 3. Influence of glucose on the expression of PDX-1 and CK2 in MIN6 cells and on CK2 kinase activity. (a) Expression of PDX-1 and CK2 subunits in MIN6 cells. MIN6 cells were treated with different glucose concentrations (0–25 mM) for 4 h. After incubation, cell extracts were analyzed on a 12.5% SDS polyacrylamide gel and transferred to a PVDF membrane. PDX-1 (polyclonal rabbit antibodies), CK2α rabbit antibody #26, α′ rabbit antibody #30, β-mouse monoclonal antibody 6D5 and α-tubulin mouse monoclonal antibody clone DM1A as a loading control were detected with the respective antibodies. Protein bands were visualized by the ECL Lumilight system of Roche Diagnostics (Mannheim, Germany). (b) Quantification of CK2 subunits after treatment of MIN6 cells with various concentrations of glucose. The experiment described in *a*

Germany, 1:200) were used. After each incubation step, the sections were washed with PBS, pH 7.4. For nuclear staining, the sections were incubated with bisbenzimide (2 μg/ml) for 5 min. The sections were then washed with PBS, pH 7.4 and flushed in *aqua dest* before they were covered with glycerol chelatine for fluorescence microscopy using a BZ-8000 microscope (Keyence, Osaka, Japan). For co-localization of PDX-1 and CK2, frozen sections of pancreatic mouse islets were prepared for double immunofluorescence staining using PDX-1 guinea pig antiserum (1:200) and an antibody against CK2α (rabbit, 1:100). Nuclei were stained with bisbenzimide (2 μg/ml). Goat-anti-rabbit-Cy3 (1:400) and goat-anti-guinea pig ALEXA-Fluor™ 488 (1:200) were applied as secondary antibodies.

2.7. Duolink® *in situ* proximity ligation assay

To detect the interaction between PDX-1 and CK2α as well as CK2β, we utilized the Duolink® *in situ* Proximity Ligation Assay (PLA, Olink Bioscience, Uppsala, Sweden) according to the manufacturer's protocol with MIN6 cells using antibodies against PDX-1 (polyclonal rabbit antibody 1:100 or mouse monoclonal antibody clone 267712, 1:50), CK2α (mouse 1A5, 1:10 or rabbit antibody #26, 1:100) and CK2β (rabbit antibody #32, 1:100). Detection of the interaction signals was carried out by red fluorescence imaging performed on an Axioskop fluorescence microscope (Zeiss, Jena, Germany). Quantification of PLA dots was performed by counting single dots in 50 cells of three different areas (total number of cells 150) on the cover slips.

2.8. Statistical analysis

Differences in kinase activity (Fig. 3c) were analyzed by the Student's *t*-test. In all tests, $p < 0.05$ was considered significant. Data are presented as mean ± S.D. of three individual experiments. All statistical tests were performed using Excel version 2003 for Windows (Microsoft, www.microsoft.com/germany).

3. Results

PDX-1 was recently identified as a substrate for protein kinase CK2 (Meng et al., 2010a). So far nothing is known about CK2 in pancreatic islets. In the present study, we attempted to analyze whether there is a direct interaction of PDX-1 and CK2 in pancreatic β-cells. First, we analyzed the localization of PDX-1 in pancreatic mouse islets. For this purpose, frozen sections of mouse pancreas were immunohistochemically stained with antibodies against PDX-1 or CK2α. Antibodies against insulin, somatostatin and glucagon were used for the identification of the islet cell type, whilst bisbenzimide was used for nuclear staining. We found PDX-1 to be localized only in the β- and δ-cells and not in the α-cells of the pancreas (Fig. 1a). To study a possible interaction of PDX-1 with CK2, we performed a double immunohistochemical staining of frozen sections of adult mouse pancreas using PDX-1 antiserum and an antibody against CK2α. PDX-1 as well as the CK2α were found in the nucleus of the islet β-cells. By merging the green and red fluorescence signals, we found a yellow staining indicating that PDX-1 co-localized with CK2α in primary mouse islets (Fig. 1b).

To get an idea about the expression level of CK2 in mouse pancreas and to support our immunofluorescence studies by a biochemical approach, we isolated the pancreas from one C57BL/6 mouse that had unlimited access to food and water. Cells were extracted and 50 μg or 100 μg of proteins in a total cell extract were analyzed on an SDS polyacrylamide gel followed by a Western blot with CK2 subunit-specific antibodies. As shown in Fig. 1c, all three CK2 subunits were expressed in the mouse pancreas. Next, we asked whether we could detect complexes between PDX-1 and CK2 in mouse pancreas extracts. Therefore, mouse pancreas extracts were incubated with a PDX-1-specific antibody. Immune complexes were analyzed on an SDS polyacrylamide gel followed by Western blot with CK2 specific antibodies. As shown in Fig. 1d, small amounts of CK2α were co-immunoprecipitated with PDX-1. These results support our initial results about an interaction of CK2 with PDX-1 in the mouse pancreas.

The Duolink® *in situ* proximity ligation assay (PLA) is an immunohistological method for the detection of protein-protein interactions (Weibrecht et al., 2010). This assay makes use of two different secondary antibodies which are coupled to a short single stranded DNA. When these two secondary antibodies bind to their corresponding primary antibodies, which are bound to closely associated proteins, the DNA strands can be linked by the addition of circle forming DNA oligonucleotides. These oligonucleotides are then amplified by a rolling circle amplification. The amplified DNA is detected with a fluorophore. This method generates bright spots when the two proteins are in close contact with each other. To study the interaction of PDX-1 with CK2α we incubated frozen mouse islets with antibodies against PDX-1 and antibodies against CK2α and performed a PLA assay. We found bright spots for co-localized PDX-1 and CK2α whereas with probes incubated with antibodies against CK2α or PDX-1 alone or with the PLA probes alone, only very few signals were detected (Fig. 2a). The dots were counted for 50 cells in three different areas of the cover slip. The quantification is shown in Fig. 2b. These data support the previously described immunofluorescence analysis about a co-localization of CK2α and PDX-1.

Since we never succeeded under *in vivo* conditions with mice in reaching blood glucose concentrations lower than 75 mg/dl, we decided to continue these analyses with MIN6 cells which have been shown to behave like primary β-cells in response to variations in the glucose concentration (Ishihara et al., 1993). In order to study the influence of various glucose concentrations on the expression of PDX-1 and on the three subunits of CK2, we incubated MIN6 cells for 4 h either in the absence of glucose or in 2.5 mM, 5 mM and in 25 mM glucose concentration (Fig. 3). Cell extracts were loaded on an SDS polyacrylamide gel followed by Western blot analysis with PDX-1 and CK2 specific antibodies. As shown in Fig. 3a, there seems to be no significant difference in the protein expression level of PDX-1 and the CK2 subunits at all glucose concentrations used in our study. Three different experiments were performed and the protein bands quantified. According to Fig. 3b, variations in the amount of CK2 subunits were not statistically significant. Thus, glucose does not seem to influence the protein level of PDX-1 and CK2 in pancreatic β-cells.

Next, we asked whether glucose might influence CK2 activity. Therefore, we repeated the experiment described above but after lysis of the cells, CK2 kinase activity was measured using the CK2 specific substrate peptide with the sequence RRRDDDSDDD (Kuenzel and Krebs, 1985). We found a concentration dependent increase in the protein kinase activity of CK2. The experiment was repeated three times (Fig. 3c). Moreover, using Student's *t*-test, we found that the increase in the kinase activity was highly significant for 2.5 and 5 mM glucose. Interestingly, we found a reduced increase in the kinase activity at a concentration of 25 mM glucose

was performed 3 times and the density of the protein bands was quantified. Statistical analysis revealed that the variations in the band intensities were not significant. (c) Influence of glucose on CK2 kinase activity. MIN6 cells were treated with different glucose concentrations (0–25 mM) for 4 h. Kinase activity of CK2 in the cell extracts was measured with the synthetic CK2 specific peptide substrate, RRRDDDSDDD, in the presence of [^{32}P] γATP. Results from three individual experiments are shown. * Significantly different from cells incubated with 0 mM glucose, $p < 0.05$. ** Significantly different from cells incubated with 0 mM glucose, $p < 0.01$.

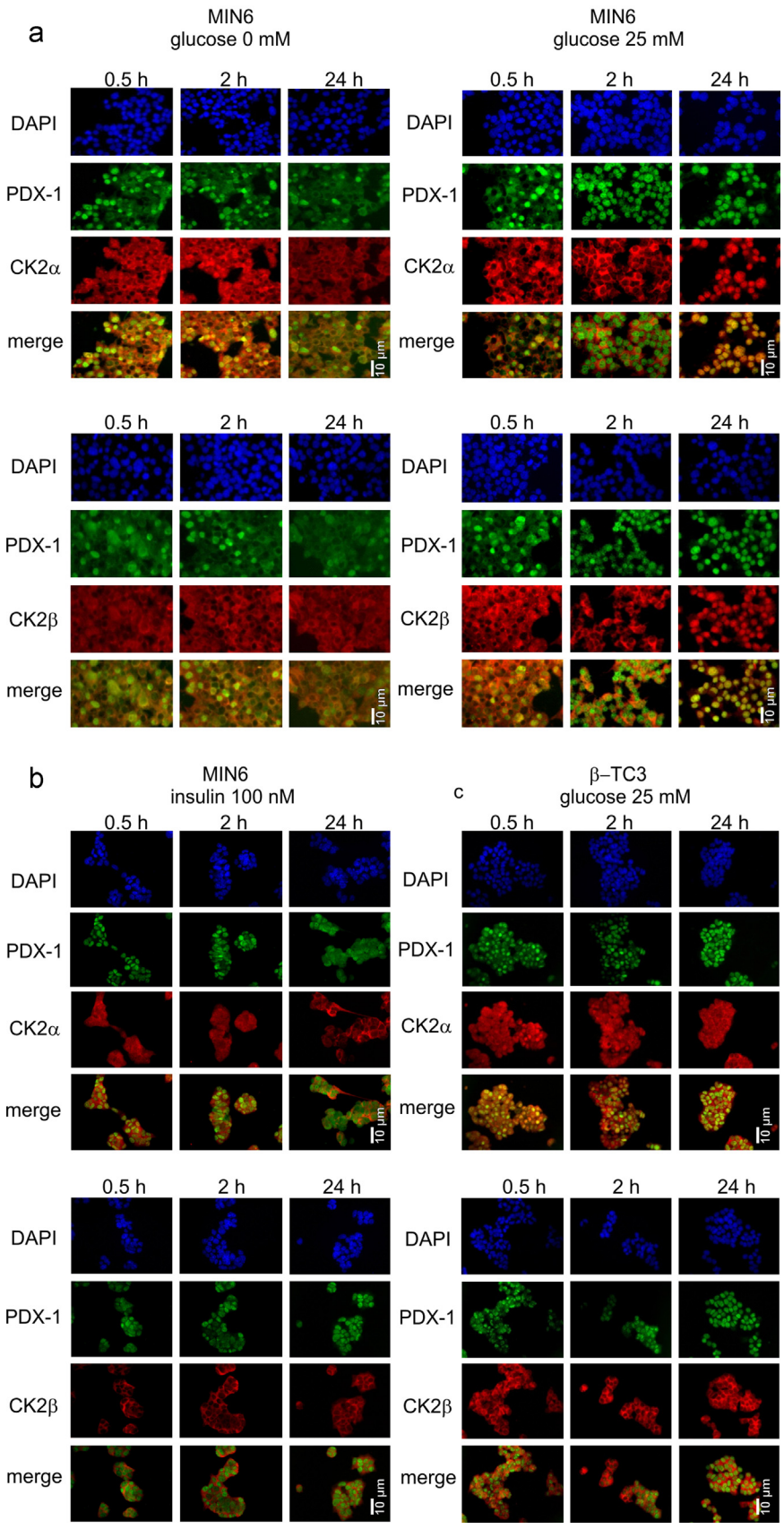

Fig. 4. Subcellular localization of PDX-1 and CK2 subunits after stimulation with glucose or with insulin. (a) MIN6 cells were cultured on coverslips and incubated in glucose-free DMEM for 8 h, before incubation with 0 mM or 25 mM glucose. After 0.5, 2 and 24 h, cells were stained with mouse monoclonal antibodies clone 267712 against PDX-1, CK2α (rabbit antibody #26), CK2β (rabbit antibody #32) and DAPI for nuclei staining. As secondary antibodies, ALEXA-Fluor™ 488 or ALEXA-Fluor™ 594 were used. Immunofluorescence was analyzed using a Zeiss Axioskop fluorescence microscope (Zeiss, Jena, Germany). (b) MIN6 cells were treated with 100 nM insulin instead of glucose and the subcellular localization of PDX-1 and CK2α and CK2β was analyzed as described above. (c) Glucose insensitive β-TC3 cells were treated with 25 mM glucose and the subcellular localization of PDX-1 and CK2α and CK2β was analyzed as described above.

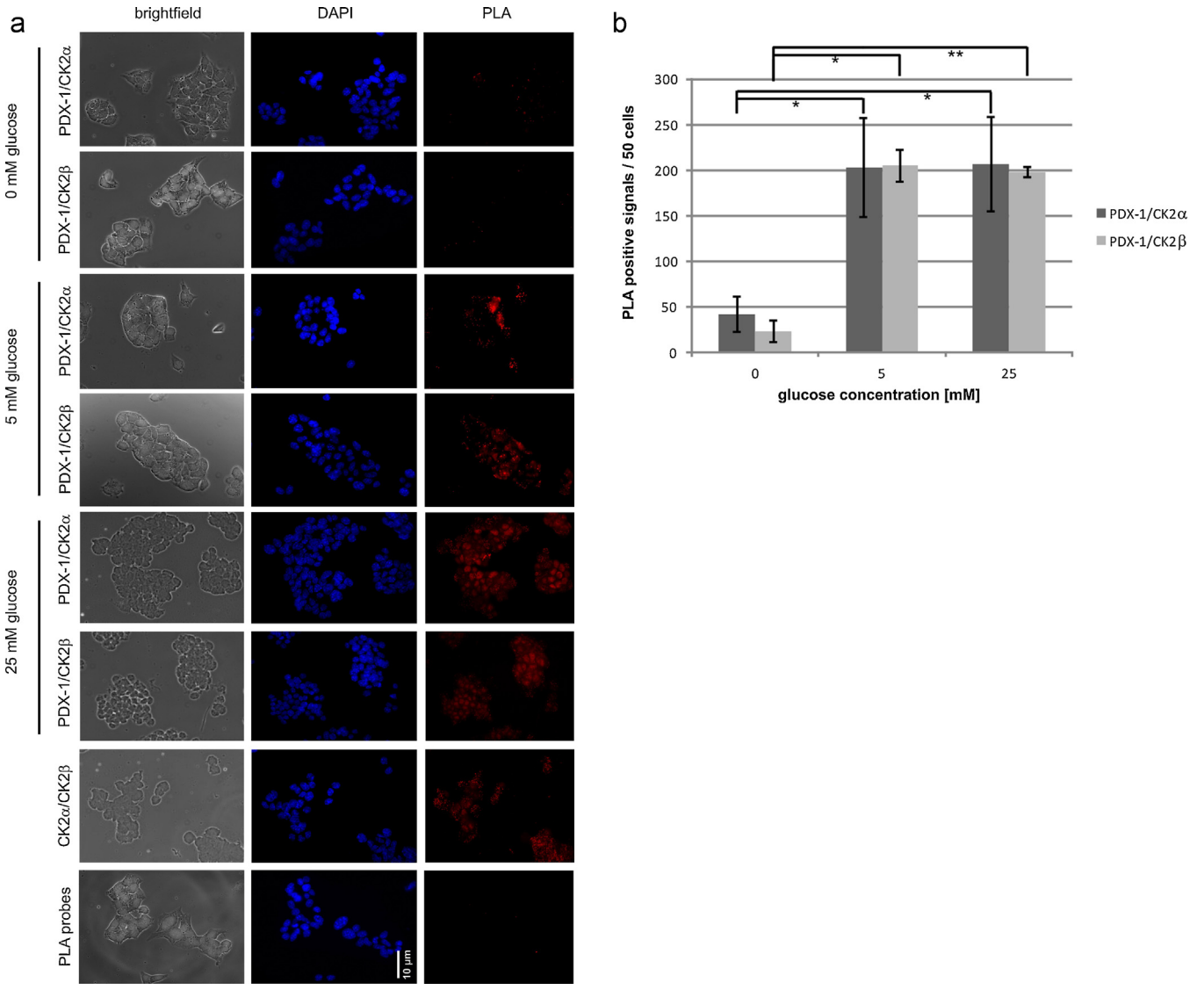

Fig. 5. Duolink® *in situ* Proximity Ligation Assay (PLA) of PDX-1 and CK2 in MIN6 cells. (a) MIN6 cells were incubated with 0 mM, 5 mM and 25 mM glucose for 4 h after 8 h starvation. Cells were subjected to Duolink® *in situ* Proximity Ligation Assay using antibodies against PDX-1, CK2α and CK2β. For positive control, a double staining of CK2α and CK2β was performed, for negative control a single staining with the PLA probes was done. Immunofluorescence was analyzed using a Zeiss Axioskop fluorescence microscope (Zeiss, Jena, Germany). Signals were counted in 50 cells of three different areas, respectively (total number of cells 150). Quantification is shown at the bottom of the figure. (b) Single dots were counted in 50 cells of three different areas (total number of cells 150). Statistical analysis was performed by using Student's *t*-test. * Significant difference $p < 0.05$, ** significant difference $p < 0.01$.

compared to 5 mM, which turned out to be also significant when compared to 0 mM glucose.

There are contradictory reports about the subcellular localization of PDX-1 and its glucose dependent shuttling from the cytoplasm into the nucleus. Therefore, we analyzed whether PDX-1 indeed shuttles and whether CK2 might also translocate into the nucleus depending on the glucose concentration. MIN6 cells were cultured on coverslips in glucose-free medium for 8 h. Cells were then further incubated with 0 mM or 25 mM glucose for 0.5, 2 or 24 h (Fig. 4a). Cells were stained for PDX-1, for CK2α or CK2β. Nuclei were stained with DAPI. In the absence of glucose, PDX-1 was found in the cytoplasm as described above. To our surprise, CK2α and CK2β were also located in the cytoplasm. The merge of the red CK2 subunit fluorescence signals with the green PDX-1 signal revealed a yellow colour for the co-localized PDX-1 and CK2 subunits. After incubation of the cells with 25 mM glucose for 2 and 24 h, PDX-1 was more and more located in the nucleus (Fig. 4a). After 24 h, all cells exhibited a nuclear signal for PDX-1. The same relocation was also found for the CK2 subunits although the nuclear localization seemed to be retarded compared to PDX-1. Thus, these results showed a glucose- dependent shuttling of PDX-1 from the cytoplasm to the nucleus. Most interestingly the same glucose-dependent relocalization was also found for the CK2 subunits. In order to analyze whether this translocation is specific for the glucose sensitive MIN6 cells, we repeated the experiment with embryonal endothel cells (eEND) (data not shown) with the prostate carcinoma cells LNCap (data not shown) and with the glucose insensitive β-TC3 cells. Fig. 4c shows that PDX-1 was found always located in the nucleus, whereas the CK2 subunits were always located in the cytoplasm and in the nucleus with no alteration upon glucose treatment.

We have previously shown that insulin stimulates CK2 kinase activity (Meng et al., 2010b). Therefore, it was an interesting question whether insulin might also regulate the translocation of CK2 from the cytosol to the nucleus. MIN6 cells were hence treated with 100 nM insulin instead of glucose and the subcellular localization was analyzed by immunofluorescence. As shown in Fig. 4b PDX-1 was rapidly translocated into the nucleus upon insulin

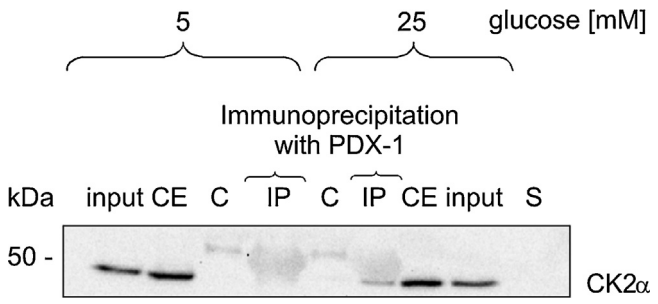

Fig. 6. Co-immunoprecipitation of PDX-1 and CK2. MIN6 cells were incubated in DMEM with 5 mM or 25 mM glucose for 24 h. Four mg of cell extracts was precleared twice with a mixture of protein A sepharose beads and CL 4-B agarose beads over a period of 1 h. The supernatants were incubated with a rabbit PDX-1 antibody for 2 h. The immunoprecipitated proteins were separated by 12.5% SDS polyacrylamide gel electrophoresis, transferred to a PVDF membrane and analyzed by Western blot with CK2α specific antibodies 1A5. Input: 5% of cell extracts were used in the experiment, CE: cell extract (100 μg), C: precipitate with protein A sepharose alone as control, IP: immunoprecipitate, S: sepharose.

treatment whereas, the CK2 subunits remained in the cytosol which excludes the notion that insulin might be responsible for the translocation. Furthermore, these results support our observation that PDX-1 and CK2 subunits translocate into the nucleus independently.

In order to support our co-localization experiment, we performed a PLA analysis. MIN6 cells were incubated in the absence of glucose for 8 h and then further incubated in the absence of glucose or in the presence of 5 mM or 25 mM glucose for 4 h. A PLA analysis was performed with PDX-1 or CK2 specific antibodies. The result of this analysis is shown in Fig. 5. We found bright spots for CK2α/PDX-1 as well as for CK2β/PDX-1 interactions only after treatment of the cells with 5 mM or with 25 mM glucose. Signals in 50 cells of three different areas of the cover slip were counted and the quantification is shown in Fig. 5b. In the absence of glucose, no such signals were observed. As a positive control we also analyzed the interaction of CK2α with CK2β which also gave bright spots. The PLA probe alone was negative as expected. Thus, we have shown that PDX-1 interacts with CK2 in the presence of high glucose concentrations.

From the results described above, we knew that PDX-1 and the CK2 subunits are co-localized in the cytoplasm and in the nucleus depending on the glucose concentration. To directly show binding of PDX-1 to CK2, we performed co-immunoprecipitation experiments from MIN6 cells which were cultured in the absence or in the presence of 5 mM or 25 mM glucose. After lysis of the cells, PDX-1 was immunoprecipitated with rabbit anti PDX-1 antibody. The immunoprecipitates were analyzed on an SDS-polyacrylamide gel followed by Western blot. The blot was developed with a CK2α specific antibody. As shown in Fig. 6, PDX-1 co-immunoprecipitated with CK2α only under high glucose conditions. Thus, these results demonstrated that PDX-1 and CK2α bind to each other only under high glucose conditions.

4. Discussion

The β-cells of the pancreatic islets are solely responsible for the transcription, translation and secretion of insulin in response to an increase in extracellular glucose concentration. A number of different factors are implicated in the regulation of these processes, perhaps the most crucial factor in β-cells is the duodenal homeobox-1 (PDX-1) protein. PDX-1 is a β-cell specific transactivator of the insulin and somatostatin genes. It binds to the promoter region of the target genes and recruits complexes of proteins that participate in the regulation of transcription of these genes. DNA binding and association with co-activators for transcription occur in the nucleus of pancreatic β-cells (Stanojevic et al., 2005; Mosley et al., 2004; Babu et al., 2008) and therefore it is believed that PDX-1 functions solely in the nucleus. However, there are controversial reports about the localization of PDX-1 either in the nucleus or in the cytoplasm where a shuttling was described depending on the glucose concentration (Macfarlane et al., 1999).

Since it turned out to be impossible to further decrease blood glucose concentration in mice, we decided to continue our study with MIN6 cells which were shown to behave like primary β-cells in response to variations in the glucose concentration. After cultivation of MIN6 cells in the absence of glucose for 8 h, PDX-1 was to some extent absent from the nucleus. By adding 2.5 mM (not shown), 5 mM (not shown) and 25 mM glucose, we observed a time-dependent shuttling of PDX-1 from the cytoplasm to the nucleus. To our surprise, in the absence of glucose, CK2 was found in the cytoplasm similar to PDX-1. We observed a time and glucose dependent shuttling of CK2 from the cytoplasm to the nucleus.

CK2α contains a classical nuclear localization motif (NLS) in its amino acid sequence, whereas, CK2β lacks such an NLS signal (Martel et al., 2001). Only few conditions are so far reported about a translocation of CK2 in eukaryotic cells. Several years ago using live cell imaging, it was shown that the CK2 subunits shuttle from the cytoplasm to the nucleus shortly after synthesis. It was also shown that CK2β enters into the nucleus much slower than CK2α (Filhol et al., 2003; Theis-Febvre et al., 2005). Moreover, movement into the cell nucleus is not uniform for each subunit indicating that CK2 free subunits, CK2α bound to CK2β or CK2 subunits bound to other cellular partner molecules and CK2β in a holoenzyme excludes CK2 from the nucleus (Filhol et al., 2003). Heat shock of prostate cancer cells leads to shuttling of CK2 from the cytosol and the nucleus to the nuclear matrix (Davis et al., 2002). It was further shown that androgen deprivation in rats leads to loss of CK2 from the nuclear matrix. Androgen stimulation of castrated rats leads to an increase in CK2 activity in the nuclear matrix fraction (Tawfic and Ahmed, 1994). However, nothing was known about the subcellular localization of CK2 in pancreatic islet cells. As shown here, in the absence of glucose CK2α and CK2β are mainly located in the cytoplasm. Upon incubation with glucose, both CK2 subunits are more and more located in the nucleus. During our intensive analyzes of the cytoplasmic nuclear transport, we noticed that CK2 subunits shuttle more slowly than PDX-1 from the cytoplasm to the nucleus. Moreover, the experiments with insulin treatment of the cells revealed that PDX-1 translocated rapidly into the nucleus whereas, the CK2 subunits remained in the cytoplasm. This observation supports the idea that either protein, may shuttle without the other one. This idea is further supported by the observation that PDX-1 only bound to CK2α under high glucose conditions. There is already some indication about regulation of the carbohydrate metabolism by CK2 either by regulation of the insulin production and secretion, regulation of the insulin receptor or regulation of enzymes implicated in carbohydrate metabolism (for review see: Al-Quobaili and Montenarh, 2012). In the present study however, we show for the first time a weak but significant influence of glucose on CK2 activity. By using co-immunoprecipitation as well as by the highly sensitive Duolink® *in situ* PLA assay we further show that CK2 and PDX-1 only bind to each other under high glucose conditions. To our knowledge, this is the first time a glucose dependent binding of CK2 to other proteins is shown. According to our present results, CK2 turns out to be glucose sensitive at least in pancreatic β-cells similar to the glucose sensitive transcription factor PDX-1. The observation that PDX-1 activity as a transcription factor is regulated by CK2 phosphorylation and the fact that CK2 binds to PDX-1 in a glucose dependent manner suggests that CK2 functions as a co-factor of PDX-1 in the regulation of insulin production and secretion in pancreatic β-cells.

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