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Pancreatic polypeptide regulates glucagon release through PPYR1 receptors expressed in mouse and human alpha-cells☆

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ABSTRACT

Background: Plasma levels of pancreatic polypeptide (PP) rise upon food intake. Although other pancreatic islet hormones, such as insulin and glucagon, have been extensively investigated, PP secretion and actions are still poorly understood.
Methods: The release of PP upon glucose stimulation and the effects of PP on glucagon and insulin secretion were analyzed in isolated pancreatic islets. Expression of PP receptor (PPYR1) was investigated by immunoblotting, quantitative RT-PCR on sorted pancreatic islet cells, and immunohistochemistry.
Results: In isolated mouse pancreatic islets, glucose stimulation increased PP release, while insulin secretion was up and glucagon release was down. Direct exposure of islets to PP inhibited glucagon release. In mouse islets, PPYR1 protein was observed by immunoblotting and quantitative RT-PCR revealed PPYR1 expression in the FACS-enriched glucagon alpha-cell fraction. Immunohistochemistry on pancreatic sections showed the presence of PPYR1 in alpha-cells of both mouse and human islets, while the receptor was absent in other islet cell types and exocrine pancreas.
Conclusions: Glucose stimulates PP secretion and PP inhibits glucagon release in mouse pancreatic islets. PP receptors are present in alpha-cells of mouse and human pancreatic islets.
General significance: These data demonstrate glucose-regulated secretion of PP and its effects on glucagon release through PPYR1 receptors expressed by alpha-cells.

1. Introduction

Pancreatic polypeptide (PP) was initially obtained as a by-product of insulin purification from chicken pancreatic extracts and later characterized in mammals [1,2]. It is a 36 amino acid protein, mainly produced in PP-cells (also named F-cells) of the endocrine pancreas [3]; as well as by a small fraction of the exocrine pancreas, gastrointestinal tract and rat adrenal medulla [4]. The presence of PP was also reported in the central nervous system (CNS) [5,6], although this point is still controversial [7]. Protein- and fat-rich meals elicit a strong stimulatory effect on PP release [8], resulting in elevated plasma levels up to 6 h after food ingestion in humans [9]. The food intake-induced rise in PP is abolished by total pancreatectomy, indicating that the pancreas is the main source of the polypeptide [10]. Some observations suggest that PP release is mainly controlled by peripheral neural elements, the main mediator of PP release being the vagal cholinergic innervation [10,11]. Adrenergic stimulation may also promote PP release after insulin-induced hypoglycemia and exercise [12–15]. In addition, cholecystokinin secreted by intestinal L-cells increases PP levels [8,10].

PP belongs to the neuropeptide Y (NPY) family of proteins, which includes NPY, peptide YY (PYY) and PP. These proteins induce cellular responses that are mediated through the Y family of inhibitory G-protein-coupled receptors (Y1-Y5, y6) [16]. NPY proteins exhibit a wide range of affinities for the different Y receptors, PP exhibiting higher affinity for Y4 (also named PPYR1) and lower for Y5 [17]. PPYR1 has been detected in the CNS in both rodents [18–21] and humans [22,23] by *Northern blot*, receptor autoradiography, in situ hybridization, histoimmunochemistry, and RT-PCR. *Northern blot* expression studies suggest that a wide range of peripheral tissues may also contain PPYR1 [20–24]. Presence of PPYR1 mRNA has been reported in human total pancreas preparations, containing both exocrine and endocrine fractions [22,24], although not in rodent pancreas.

PP has recently gained interest due to the major role this peptide plays in the control of appetite. Indeed, peripheral PP acts as a long term satiety signal both in mice and humans [25,26] and the islets of obese *ob/ob* mice contain relatively few PP-producing cells [27]. Studies in knockout mice lacking PPYR1 revealed that these receptors are

Abbreviations: CNS, central nervous system; FACS, Fluorescence Activated Cell Sorting; PP, pancreatic polypeptide; PPYR1, pancreatic polypeptide receptor

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necessary for the food-intake inhibitory properties of PP [28]. However, the mechanisms involved in the inhibitory effects of PP on food intake have not been clarified. Such anorexic response might involve central mechanisms through specific brain nuclei and/or peripheral circuits associated with vagal afferent pathways. Other peripheral biological actions of PP have been proposed; such as the regulation of hepatic glucose production, delay of gastric emptying, decreased intestinal motility, reduction in bile acid output and pancreatic exocrine secretion [29]; although the detailed mechanisms remain unknown.

Crosstalk between different cell types composing the endocrine pancreas participates to the fine tuning of endocrine function in the control of metabolic homeostasis. Intra-islet paracrine functions have been described for the pancreatic hormones insulin [30,31] and somatostatin [30,32,33]. In this context, we hypothesized that the effects of PP might be mediated by intra-islet paracrine regulations. The present study investigated the peripheral functions of PP in pancreatic islets. We observed expression of the PP receptor PPYR1 in the mouse and human endocrine pancreas, as well as glucose-regulated stimulation of PP secretion and its modulatory effects on glucagon release from mouse pancreatic islets.

2. Material and methods

2.1. Insulin, glucagon and pancreatic polypeptide secretion experiments

Pancreatic islets from male C57BL/6 mouse were isolated by collagenase digestion, in accordance with the Institutional Animal Ethics Committee's policies, and cultured overnight free-floating in RPMI-1640 medium before use. For static incubations, islets were washed and preincubated for 30 min in 2.8 mM glucose Krebs–Ringer bicarbonate HEPES buffer [KRBH, containing in mM: 135 NaCl, 3.6 KCl, 10 HEPES (pH 7.4), 5 NaHCO3, 0.5 NaH2PO4, 0.5 MgCl2, 1.5 CaCl2, 0.1% bovine serum albumin]. Then, batches of 3–4 islets were hand-picked and incubated for 60 min at 37 °C at basal (2.8 mM) or stimulatory (15 mM) glucose concentrations. For the measurements of PP release, batches of 7 islets were hand-picked and incubated for 60 min at 37 °C at basal (2.8 mM) or stimulatory (15 mM) glucose concentrations. Insulin, glucagon and PP concentrations were measured by Mouse Endocrine Multiplex Immunoassay from Linco (Millipore, Billerica, MA, USA).

2.2. Source and species of pancreatic polypeptide

Mouse pancreatic polypeptide (GenBank: EDL34086.1, 100 aa), used for stimulation of isolated mouse pancreatic islets, was synthesized by Proteomics Core Facility in Pompeu Fabra University-Centre for Genomic Regulation (Barcelona, Spain).

2.3. Perifusion of mouse pancreatic islets

Islet perifusion was carried out using 200 hand-picked islets per chamber, thermostated at 37 °C (Brandel, Gaithersburg, MD, USA). The flux was set at 250 μL/min and fractions were collected every minute, after a 30 min preincubation period at basal glucose (2.8 mM), in tubes containing 500 KUI/mL aprotinine (Applichem). At minute 18, PP (10 nM) was added to the perifusion buffer. At the end of the perifusion period, islets were collected from the chamber and acid-EtOH extracts were prepared to measure the remaining islet glucagon contents. Glucagon levels were determined by RIA kit according manufacturer's instructions (Millipore GL-32K). Glucagon secretion was expressed as pg/mL.

2.4. Western blotting

Tissues were obtained from C57/BL6J male mice (Charles River) and stored at −80 °C. Frozen samples were dounce-homogenized in 30 volumes of lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 1 g/mL aprotinin, 1 μg/mL leupeptine, 1 μg/mL pepstatin, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 100 mM sodium fluoride, 5 mM sodium pyrophosphate, and 40 mM beta-glycerolphosphate) plus 1% Triton X-100. After 10 min at 4 °C, samples were centrifuged at 16,000 *g* for 30 min to remove debris and protein concentrations in the supernatants were determined by DC-micro plate assay (Bio-Rad, Madrid, Spain). Equal amounts of lysates were mixed with denaturing 5x Laemmli loading buffer and boiled for 5 min. Samples with equal amounts of total protein (20 μg per lane) were separated in 10% sodium dodecyl sulfate-polyacrylamide gel before electrophoretic transfer onto nitrocellulose membrane (Bio-Rad, Spain). Membranes were blocked for 1 h at 21 °C in Tris-buffered saline (TBS) (100 mM NaCl, 10 mM Tris, pH 7.4) with 0.1% Tween-20 (TBS-T) and 5% non-fat milk. Afterwards, membranes were incubated overnight with the primary antibodies, rabbit polyclonal antibody against rat PPYR1 (SA-644, 1:200; Enzo Life Sciences, Farmingdale, NY, USA) and mouse monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:5,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Bound primary antibodies were detected with horseradish peroxidase-conjugated antibodies to rabbit or mouse antibodies (Pierce, diluted 1:5000) and visualized by enhanced chemiluminescence detection (West-Femto-SuperSignal, Pierce). Representative cropped immunoblots for display were processed with Adobe Photoshop 7.0.

2.5. FACS sorting of pancreatic islet cells

Pancreatic islets from male C57BL/6 mouse fed *ad libitum* were isolated by collagenase digestion, in accordance with the Institutional Animal Ethics Committee's policies, and cultured free-floating overnight in RPMI-1640 medium before use. Isolated islets were dispersed into individual cells by treatment with trypsin, and the freshly dissociated cells were subjected to Fluorescence Activated Cell Sorting (FACS) using a FACS-IV instrument. An argon laser illuminated the cells at 488 nm, and emission was monitored at 510–550 nm. This technique yields two populations of cells, one enriched in alpha cells and one enriched in beta cells.

2.6. Quantitative RT-PCR

Total RNA was extracted from FACS-sorted islet cells by use of the acid guanidinium phenol preparation TRIZOL (#15596-026, Invitrogen, CA, USA) following the manufacturer's instructions. Total RNA obtained from sorted islet cells was reverse transcribed by Superscript III reverse transcriptase (#18080, Invitrogen). The oligonucleotides (Sigma Aldrich, MO, USA) used to perform subsequent cDNA amplifications by quantitative RT-PCR were: 61-mPPYR1.for: 5′-GCT TCC TAG CCA GGA CTT GGT-3′, 185-mPPYR1.rev: 5′-AAA GGG CCT ACT TCA GAG ATG C-3′, 46-mGLUC.for: 5′-GGA CTC CCT CTG TCT ACA CCT GTT-3′, 146-mGLUC.rev: 5′-GCA CCA GCA TTA TAA GCA ATC CA-3′, 104-mINS.for: 5′-CTG GTG GGC ATC CAG TAA CC-3′, 210-mINS.rev: 5′-GGG TAG GAA GTG CAC CAA CAG-3′. Hypoxanthine-guanine phosphoribosyl transferase (HPRT) mRNA was analyzed as an internal control by using oligonucleotides 711-mHPRT.for: 5-GTT GGA TAT GCC CTT GAC TAT AAT GAG TA-3′ and 790-mHPRT.rev: 5′-TTG GCT TTT CCA GTT TCA CTA ATG-3′. Quantitative determination of PPYR1, insulin, glucagon and HPRT mRNA levels was performed simultaneously in triplicate by using Power SYBR® Green PCR Master Mix, (#4367659, Applied biosystems, CA, USA). qRT-PCR and data collection were performed on the ABI Prism 7900HT system. All quantifications were normalized to the endogenous control (HPRT).

2.7. Preparation and cryostat sectioning of mouse and human pancreatic tissue

C57/BL6J male mice were anesthetized by *i.p.* administration of sodium pentothal 100 mg/kg body weight and immediately perfused with

freshly prepared 4% paraformaldehyde (PFA). After perfusion, the pancreas was dissected, rinsed in phosphate buffered saline (PBS) and kept in 4% PFA overnight. Afterwards, pancreatic tissue was washed with PBS. Mice samples were kept overnight in 15% sucrose, embedded in gelatin (3 h, 37 °C), transferred to cryomolds, frozen for 1 min in isobutyryl (pre-cooled at −80 °C) and cryostat-sectioned (10 μM alternate sections). Sections were kept at −80 °C.

Human samples, with informed family consent and approval by the Ethics Committee, were kindly provided by Institut d'investigacions Biomèdiques August Pi i Sunyer (IDIBAPS, Barcelona). These samples were obtained postmortem from the duodenal part of the pancreas from healthy donors and immediately frozen at −80 °C.

2.8. Immunohistochemistry

Human pancreatic sections kept at −80 °C were fixed 2 h with 4% PFA. Mouse pancreatic sections, of already fixed mice pancreas, were allowed to defreeze. Sections were washed with phosphate buffered saline containing 0.1% Triton (PBTx) and blocked with PBTx containing 10% goat serum (GS/PBTx) (1 h, 21 °C). Primary antibodies were incubated in GS/PBTx (overnight, 4 °C). Sections were washed with PBTx and secondary fluorescent antibodies were incubated in GS/PBTx (2 h, 21 °C). From this point, sections were always protected from light. Sections were washed with PBTx several times and mounted with mounting medium (S3023, DakoCytomation, Dako).

2.9. Antibodies

2.9.1. PPYR1 primary antibodies

Three different antibodies were assayed for immunodetection of PPYR1: i) rabbit polyclonal antibody to rat PPYR1 (SA-644, Biomol International, US). This antibody was generated by injecting a peptide corresponding to aminoacid residues 326–340 of rat PPYR1, which is identical in mouse PPYR1. This antibody identified the protein only in mouse sections (1:100 dilution). Preadsorption control was done with the control peptide (ratios 1:1 and 1:5). ii) rabbit polyclonal antibody to the second extracellular domain of the human PPYR1 receptor (ab13371, Abcam). This antibody identified both mouse and human samples (1:100 dilution). iii) rabbit polyclonal antibody to the C-terminal domain of the human PPYR1 receptor (ab13369, Abcam). In our experimental conditions this antibody provided a fuzzy staining in the islets that did not seem to be specific.

2.9.2. Primary antibodies for endocrine pancreatic hormones

The antibodies used were: mouse anti-insulin (1:500; I-2018, Sigma), mouse anti-glucagon (1:1000; G-2654, Sigma), mouse anti-somatostatin (1:100; GTX71935, GeneTex), guinea pig antibody to rat PP (1:100 dilution; 4041–01, Millipore) for mouse samples, and goat antibody to human PP (1:100; EB06805, Everest Biotech) for human samples.

2.9.3. Secondary antibodies

Goat anti-rabbit Cy3 (111-225-144, Jackson), goat anti-mouse Cy2 (115-225-146, Jackson), goat anti-guinea pig Cy2 (106-225-003, Jackson), donkey anti-rabbit Alexa555 (A31572, Molecular Probes), donkey anti-mouse Alexa647 (A31571, Molecular Probes) and donkey anti-goat Cy2 (705-225-147, Jackson) were used as secondary antibodies. All of them were diluted to 1:500. When anti-rabbit and anti-guinea pig secondary antibodies were used, a sequential double immunostaining was done in order to avoid cross-reactivity.

2.10. Confocal microscopy and imaging

Confocal images were obtained using a Leica SP2 confocal microscope, adapted to an inverted Leica DM IRBE microscope. Tissue sections were examined with a 40× 1.25 NA oil immersion Leica Plan Apochromatic objective. Cy2, Alexa555 and Alexa647 were excited with the 488 nm line of an argon laser, the 555 nm line of a green neon laser and the 647 nm line of a helium neon laser respectively, and double or triple immunofluorescence images were taken in a sequential mode. Images were analyzed with Adobe photoshop 7.0.1.

2.11. Statistical analysis

Data are presented as the means ± S.E. as indicated. Differences between groups were assessed by two way ANOVA analysis and subsequent post-hoc Fisher test when required. Comparisons were considered to be statistically significant when the level of significance was $p < 0.05$.

3. Results

3.1. Glucose increases PP release from mouse pancreatic islets

In order to avoid potential effects contributed by differences of islet cell composition regarding their pancreatic localization, islets were isolated from the whole pancreas and distributed randomly for secretion assays. As expected, stimulation of isolated mouse islets with 15 mM glucose increased insulin secretion (14.1–fold, $p < 0.01$, Fig. 1A) and decreased glucagon release (−37%, $p < 0.05$, Fig. 1C) compared to basal conditions at 2.8 mM glucose. Under the same conditions, 15 mM glucose induced marked increase of PP release (+92%, $p < 0.05$, Fig. 1B). On average, mouse pancreatic islets secreted 6.2 ± 1.5 pg PP/islet h (4 independent experiments).

3.2. PP modulates glucagon secretion in mouse islets

Secretion experiments were performed on mouse islets to investigate the potential effects of PP on glucagon release from neighboring α-cells. We tested a wide range of PP concentrations, up to 100 nM, based on the observed paracrine effects of somatostatin in the 10–1000 nM range on glucagon release from rodent islets [34]. At 1 nM, PP decreased glucagon release by 59% ($p < 0.01$, Fig. 1D), whereas at the highest concentration (100 nM) PP exhibited the reverse effect, increasing glucagon secretion (+47%, $p < 0.01$, Fig. 1D). The inhibitory effect of physiological PP on glucagon release was confirmed in dynamic secretion experiments performed on islets perifused at basal 2.8 mM glucose in the absence and presence of 10 nM PP (Fig. 1E). In such perifusion protocol, both glucagon and insulin were measured in parallel in the same samples of islet perifusate. Whereas insulin release remained stable (Fig. 1G), glucagon secretion was reduced by 48% upon PP exposure (Fig. 1F).

3.3. The receptor PPYR1 is expressed in pancreatic islet cells

Although some studies reported expression of PPYR1 at the mRNA level in mice and humans in the CNS and some peripheral tissues (22–29), characterization at the protein level has not been documented yet. Thus, mouse tissues were isolated and PPYR1 expression was analyzed by immunoblotting, using an antibody raised against the rat PPYR1 C-terminal region, which is identical in the mouse. In pancreatic islets, a single band was observed in-between 50 and 75 kDa, in accordance with PPYR1 expected size (Fig. 2). Bands ranging from 50 to 75 kDa were found in the following brain areas: brainstem, hippocampus, hypothalamus and striatum (Fig. 2A). While hippocampus, hypothalamus and striatum shared a very similar pattern of bands, the brainstem exhibited 5 different bands. In brainstem, the lowest band (approx. 54 kDa) corresponded to the one found in pancreatic islets (Fig. 2A), whereas the adjacent upper band (approx. 60 kDa) corresponded to the strongest band in hippocampus, hypothalamus and striatum (Fig. 2A). Some of those bands were also found in two additional peripheral tissues, i.e. the skeletal muscle (Fig. 2B) and the adrenal glands (Fig. 2A). We noticed absence of immunoreactivity in the liver, kidney and seminal glands (Fig. 2B). These results show that

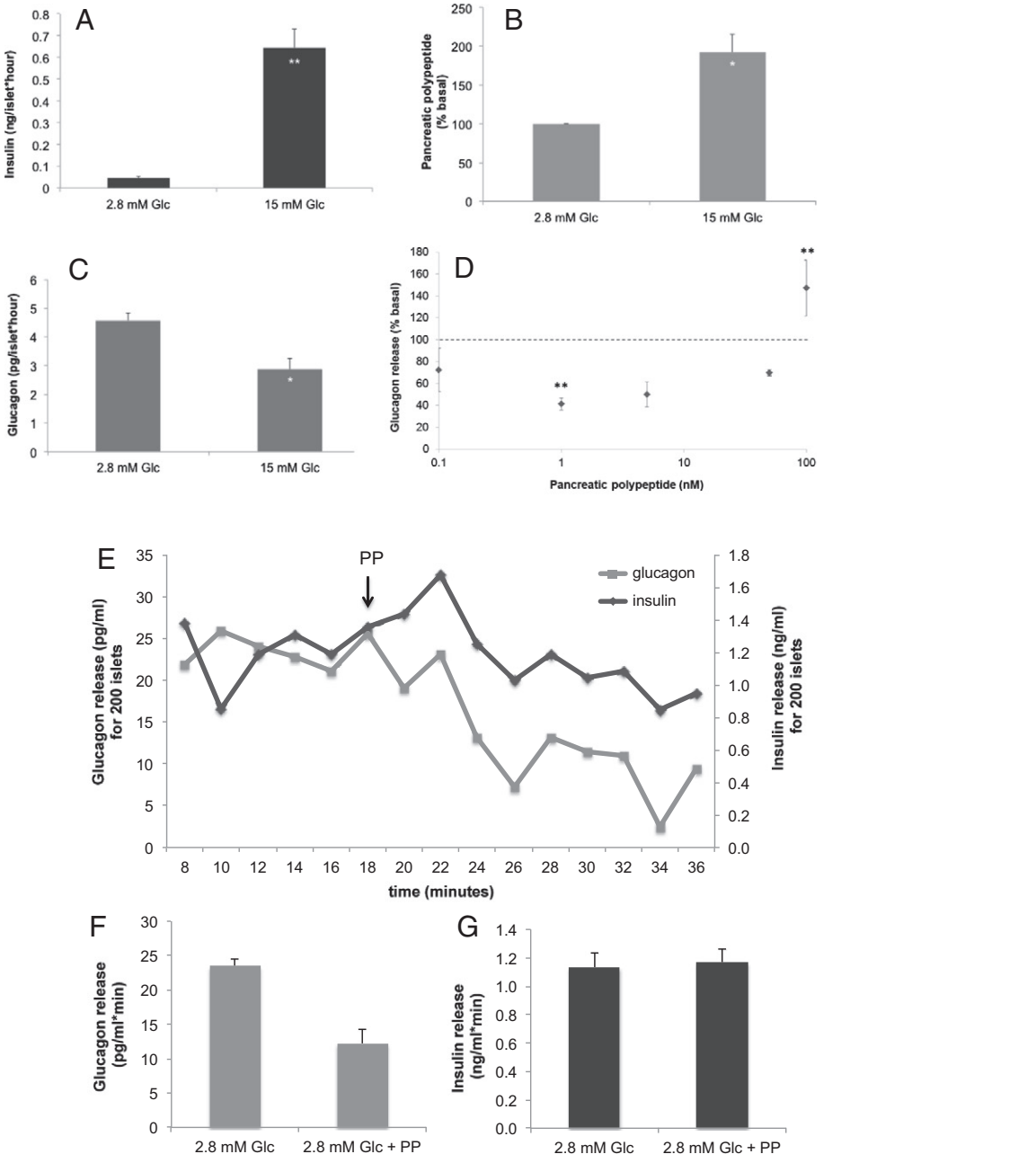

Fig. 1. Effect of glucose and PP on pancreatic hormone release. A–C) Effects of 15 mM glucose on the release of insulin (A), PP (B), and glucagon (C) from isolated mouse pancreatic islets. Results are the means of 4 experiments performed in triplicate. $p < 0.05$ (*), $p < 0.01$ (**) vs. 2.8 mM glucose. D) Dose response of PP on glucagon release from isolated islets at 2.8 mM glucose. Results are the means of 5 experiments performed in triplicate. $p < 0.01$ (**) compared to the absence of PP, set as basal 100% release. E-G) Effects of addition of 10 nM PP (arrow) on glucagon and insulin secretion during perifusion (E) of isolated mouse islets at 2.8 mM glucose. Area under the curves, calculated from (E), for glucagon (F) and insulin (G) release.

PPYR1 is expressed in the endocrine pancreas and confirm PPYR1 expression in different areas of the brain associated with PP function.

Using FACS, mouse pancreatic islet cells were separated into 2 populations, *i.e.* non-ß and ß-cells. These fractions were analyzed by qRT-PCR, revealing high mRNA levels of insulin in the ß-cell fraction and of glucagon in the non ß-cell fraction (Fig. 2C). PPYR1 mRNA was present mainly in the non ß-cell fraction, and at much lower levels in the ß-cell fraction (Fig. 2C).

3.4. PPYR1 is expressed specifically in α-cells of mouse and human islets

The observed expression of PPYR1 at mRNA level in the non ß-cell fraction of pancreatic islets, prompted us to identify the cell type expressing the receptor at the protein level. Immunohistochemistry was performed on mouse pancreatic sections using the PPYR1 C-terminal-targeted antibody already characterized by immunoblotting (Fig. 2). This revealed the presence of PPYR1 at the periphery of the islets, where no ß-cells are located, and absence of PPYR1 in the exocrine parts (Fig. 3A). Next, using co-immunostaining, expression of PPYR1 was analyzed in different pancreatic islet cell types. PPYR1 expression exhibited a highly specific pattern restricted to glucagon-containing α-cells (Fig. 3Bc). Indeed, all of the glucagon-positive cells were positive for PPYR1 and vice versa ($n = 25$ islets). PPYR1 was absent in both ß-cells (Fig. 3Ba) and δ-cells (Fig. 3Bb). Pre-adsorption with PPYR1 peptide (Fig. S1a,b) confirmed the specificity of PPYR1 antibody used for immunoblotting (Fig. 2A and B) and immunohistochemistry (Fig. 3).

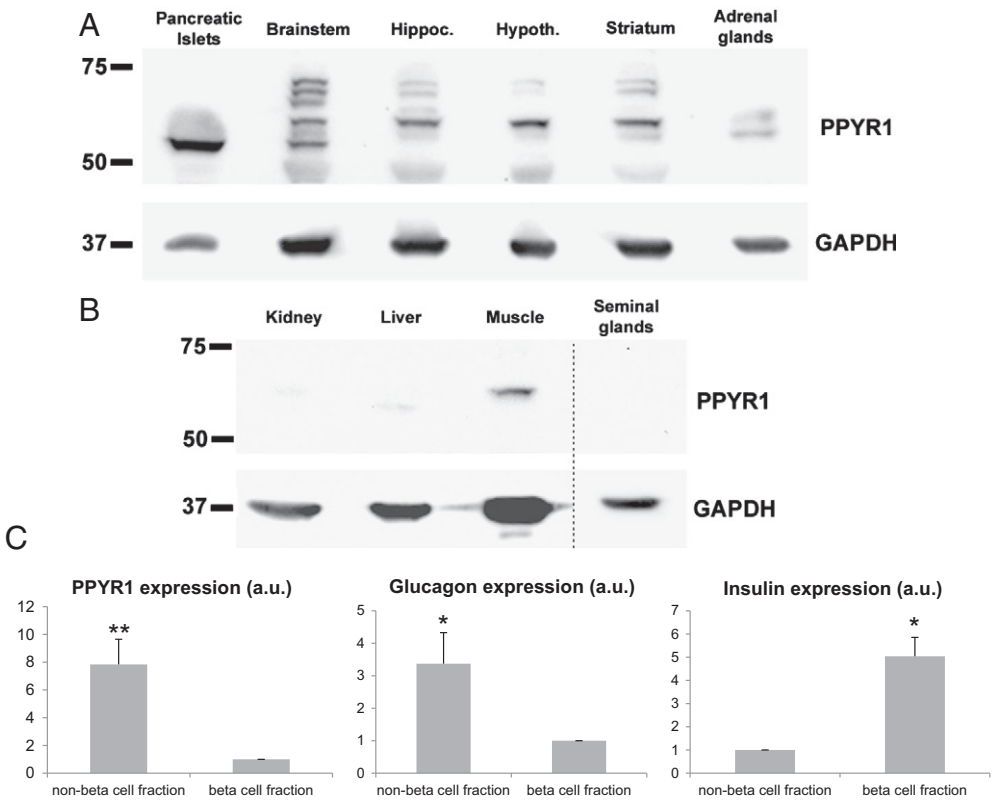

Fig. 2. PPYR1 is expressed in mouse pancreatic islets, preferentially in non-beta cells. A–B) Immunoblotting on protein extracts from isolated mouse pancreatic islets, brain areas and peripheral tissues. A band between 50 and 75 kDa is expected for PPYR1. Pancreatic islets exhibited a single band, while a set of bands between 50 and 75 kDa was obtained in brainstem, hippocampus, hypothalamus and striatum (A). Similar bands were also present in adrenal glands (A) and skeletal muscle (B). GAPDH was used as loading control. Dashed line indicates edition of the photograph to bring closer a distant lane. Hippoc: Hippocampus; Hypoth: Hypothalamus. C) Quantitative RT-PCR on mRNA for PPYR1, glucagon and insulin on mouse pancreatic islet cells following FACS. Results are the mean of 3 independent experiments. $p < 0.05$ (*) and $p < 0.01$ (**).The mRNA levels were normalized to HPRT, a.u. arbitrary units expressed as fold changes compared to the beta-cell fraction (for PPYR1 and glucagon) or non-beta cell fraction (for insulin).

Next, PPYR1 expression was assessed in human pancreatic sections, using an anti-PPYR1 antibody raised against a synthetic human protein. Immunolocalization revealed the presence of PPYR1 protein in human pancreatic islets while it was not detected in the exocrine part (Fig. 4A). PPYR1 was found in cells scattered within the islet, consistent with the spatial organization of non ß-cells in human islets [35]. Co-labeling with islet hormones revealed that PPYR1 was absent in both insulin and somatostatin positive cells (Fig. 4Ba and 4Bb, respectively), whereas present in glucagon positive α-cells (Fig. 4Bc). Of note, α-cells exhibited intermingled pattern, as previously characterized for human islets [35]. Reactivity of the anti-human PPYR1 antibody was tested on mouse pancreatic samples and provided similar pattern (Fig. S2b). In particular, the results obtained with both antibodies were consistently showing expression of PPYR1 exclusively in α-cells, indicating conserved intra-islet PP signaling in mammals.

3.5. PPYR1 is excluded from PP cells

Finally, we evaluated the possibility of an autocrine loop mediated by PPYR1 in PP-cells. Immunohistochemistry was performed to compare the expression of the receptor and its ligand in mouse pancreatic islets. Beside peripheral PP-cells, a small subset of α-cells was positive for PP (Fig. 5a), in agreement with previous studies [36,37]. Triple immunostaining allowed discrimination of PP-positive α-cells versus PP-cells *per se* (Fig. 5b). In all cases ($n = 6$ islets), PP-cells being negative for glucagon were also negative for PPYR1. The PP-cells identified in human sections were also free of PPYR1 (Fig. S3). These results demonstrate that PP-cells do not express PPYR1 and that the only islet cell type expressing PPYR1 is the α-cell.

4. Discussion

PP has recently gained interest due to its central effects on appetite. However, compared to other pancreatic hormones, PP signaling remains poorly characterized within the islet. In the present report, we demonstrate PP release upon glucose stimulation in mouse islets, as well as modulation of glucagon secretion by PP. According to such signaling, PP receptor PPY1R was found in α-cells in both mouse and human islets. Therefore, we propose that PP, through its receptor PPYR1, modulates α-cells.

Analysis of tissue distribution of PPYR1 protein in mice revealed a single band in pancreatic islets. In contrast, brain extracts exhibited a multiple band pattern, probably explained by different N-glycosylation states of PPYR1 [21,23]. Our observations are consistent with previous studies showing PPYR1 expression in rat brainstem [19] and hypothalamus [20], and PPYR1 ligand binding in rat hippocampus [38] and brainstem [18,38]. In humans, PPYR1 mRNA was detected in hypothalamus [22] and hippocampus [22,23]. The present results confirm this pattern of expression at the protein level and suggest a conserved role for PPYR1 in these CNS areas. In peripheral tissues, we found PPYR1 protein in skeletal muscle and adrenal glands, in accordance with previous results [21,23,39].

4.1. Regulation of PP release by glucose

In humans, plasma PP levels increase 30 min after food intake [40–42] or glucose load, though to higher levels in type 2 diabetes [43]. Since pancreatic islets are the source of plasma PP, we evaluated the role of glucose on PP release. At glucose concentrations resulting in

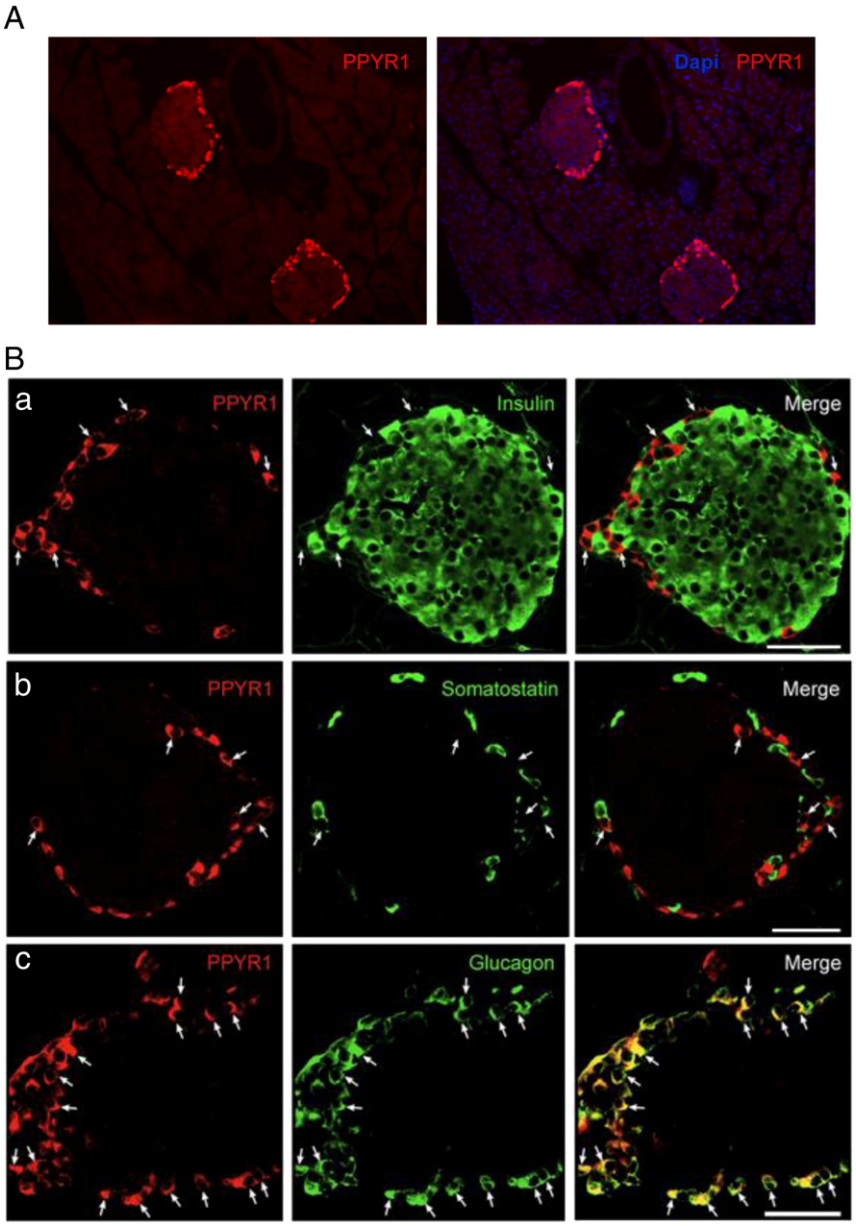

Fig. 3. Expression of PPYR1 in alpha-cells of mouse islets. A) PPYR1 immunostaining on mouse pancreatic sections. Red shows PPYR1 and blue DAPI-positive nuclei. PPYR1 expression is found exclusively within pancreatic islets. B) Double-immunostaining for PPYR1 plus (a) insulin, (b) somatostatin, and (c) glucagon in mouse pancreatic sections. Red shows PPYR1 and green insulin, somatostatin, and glucagon. White arrows point to cells expressing PPYR1. Scale bar represents 50 μm.

increased insulin secretion and reduced glucagon release, PP secretion was doubled compared to basal glucose in mouse islets. Although this represents the first demonstration of glucose-stimulated PP release in isolated islets, these data are in accordance with previous report showing that PP-cells share regulatory mechanisms with other islet cell types, being responsive to calcium and expressing both K-ATP and voltage-dependent calcium channels [44]. PP-cells can also be modulated by other hormones, for instance positively by the glucose-dependent insulinotropic polypeptide (GIP), as shown recently [43].

4.2. Regulation of glucagon release by PP

Regarding the physiological role of PP within the islet, PP might participate to the modulation of glucagon release on neighboring α-cells. Paracrine regulation of glucagon secretion, mediated by ß- and δ-cells, has been proposed. The ß-cell releases not only insulin [31,45] but also GABA [46] and zinc [47], potentially inhibiting glucagon release. Additionally, glucose promotes somatostatin release by δ-cells, inhibiting glucagon secretion through somatostatin receptors located in α-cells [32,33]. Nevertheless, the mechanisms implicated in the regulation of glucagon release remain unsolved [30]. Some previous studies pointed to PP as a modulator of glucagon release. Indeed, PP administration to rats slightly increases basal glucagon levels [48] and in the isolated perfused rat pancreas PP stimulates glucagon secretion [49]. In humans, plasma PP levels, increased after a meal, have been measured in the range of 0.1 nM [40] to 0.3 nM [41], up to 23 nM in case of a high carbohydrate meal [42]. Using this range of concentrations, we tested the effects of PP on glucagon release from isolated mouse islets. PP dose–response revealed an inverted bell-shape pattern of glucagon release, being inhibitory at physiological PP concentrations (1–10 nM) and stimulatory at supra-physiological 100 nM. Importantly, PP did not influence insulin release, showing the specificity of PP action on islet cell sub-population. Such specificity could be contributed by selective expression of PP receptors and/or by the close vicinity of the

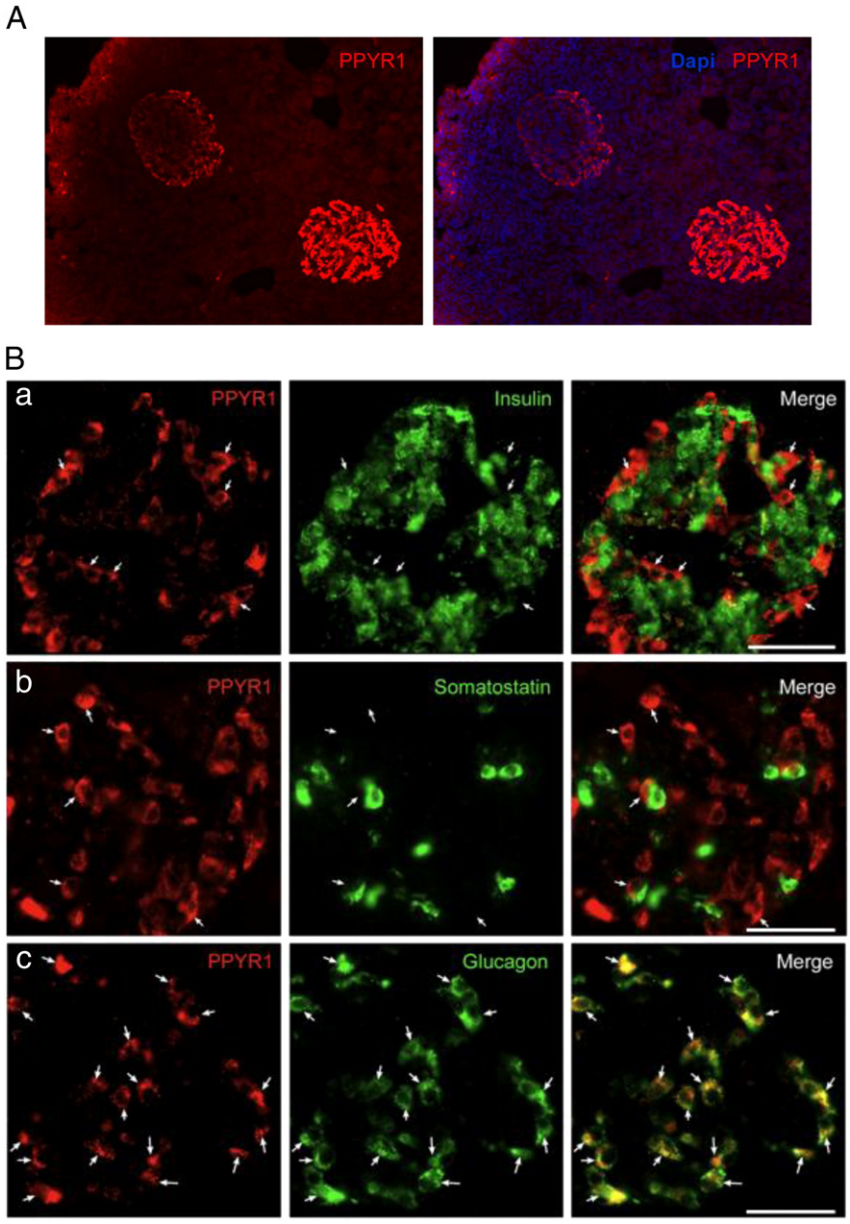

Fig. 4. Expression of PPYR1 in alpha-cells of human islets. A) PPYR1 immunostaining in human pancreatic sections. Red shows PPYR1 and blue DAPI-positive nuclei. PPYR1 expression is found exclusively within pancreatic islets. B) Double-immunostaining for PPYR1 and (a) insulin, (b) somatostatin, and (c) glucagon in human pancreatic sections. Red shows PPYR1 and green insulin, somatostatin, and glucagon. White arrows point to cells expressing PPYR1. Scale bar represents 50 μm.

responsive glucagon α-cells. It has been reported that islets in the head of the pancreas are rich in PP-cells and poor in α-cells, while those in the tail are poor in PP-cells and rich in α-cells [50]. Circulating PP would mainly activate PPYR1 receptors belonging to pancreatic islets poor in PP-cells. This might be specially relevant in humans, where pancreatic endocrine cells are in the close vicinity of vascular cells, closely associated with islet microcirculation [35].

4.3. Presence of PPYR1 in mouse and human α-cells

The mRNA analysis on sorted islet cells was substantiated by immunohistochemical approaches. Two different antibodies against PPYR1 gave consistent results, revealing PPYR1 exclusively in α-cells, both in mouse and human islets. These unprecedented results show that PPRY1 is expressed in a very specific sub-set of pancreatic cells, composing less than 1% of the whole pancreas. This might explain the very low levels of PPYR1 mRNA previously detected in human total pancreas [22,24]. Of note, immunohistochemistry showed that about 25% of the PPRY1-positive α-cells exhibited some PP immunoreactivity. Accordingly, it has been reported that PP antibodies may cross-react with PYY present in α-cells [36] and that a subpopulation of cells co-express PP and glucagon [37]. Of note, cells being positive for PP and negative for glucagon did not express PPYR1. Similar cross-reactivity of PP antibodies with some α-cells and exclusion of PPYR1 in PP-cells was observed in human islets. The absence of PPYR1 in PP-cells argues against an autocrine function in pancreatic islets.

4.4. Biological significance of a regulation of glucagon by PP

Exposure of mouse islets to 1 nM PP inhibited glucagon release. Over a 1 h period of glucose stimulation, about 6.2 pg of PP was

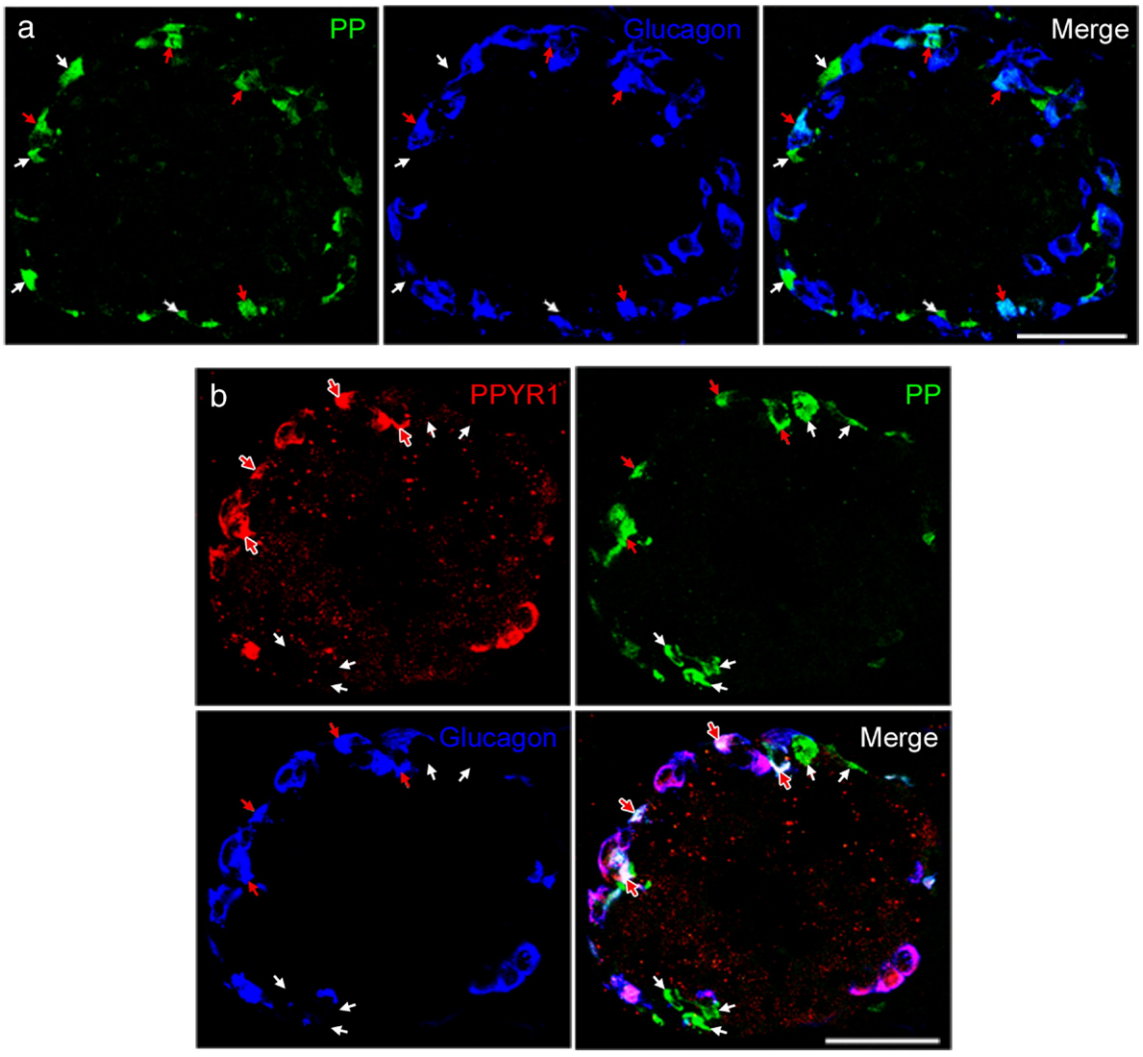

Fig. 5. PPYR1 is absent in PP-cells. (a) Double-immunostaining for PP and glucagon and (b) triple immunostaining for PPYR1, PP, and glucagon in mouse pancreatic sections. Green shows PP, blue glucagon, and red PPYR1. Red arrows point to glucagon-positive alpha-cells also positive for PP. White arrows point to cells positive for PP and negative for glucagon. Scale bar represents 50 μm.

secreted per islet. It is likely that intra-islet endogenous PP could reach active concentrations, in particular regarding action on α-cells being adjacent to PP-cells. Thus, PP released from PP-cells upon glucose stimulation would interact with PPYR1 receptors on α-cells, thereby inhibiting glucagon release. Activation of PPYR1 can modulate intracellular concentrations of cAMP and Ca^{2+} [22,51], both signaling molecules involved in the regulation of glucagon release [52,53]. The apparent paradoxical reversed effect of high concentrations of PP on glucagon release might be mediated by changes in cAMP, which is either inhibitory or stimulatory at low and high concentrations, respectively [52].

4.5. Future directions and conclusions

Several paracrine regulations within the islet have been described, such as the negative control of insulin over glucagon secretion [31] and the inhibitory effect of somatostatin on the release of insulin [33] and glucagon [32,33]. This mode of communication between the different islet cell types is thought to mediate fine-tuning of pancreatic endocrine function for the maintenance of metabolic homeostasis. Paracrine interactions are particularly important in human islets [35]. Our data add a layer of complexity by demonstrating a modulating role of PP on α-cells.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.11.005.

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