Journal of Steroid Biochemistry & Molecular Biology 115 (2009) 20–29

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Human pancreas-specific protein disulfide isomerase homolog (PDIp) is an intracellular estrogen-binding protein that modulates estrogen levels and actions in target cells

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ARTICLE INFO

Article history:
Received 6 November 2008
Received in revised form 5 February 2009
Accepted 9 February 2009

Keywords:
PDI
PDIp
Estrogen receptor
SHBG
Pancreas
Disulfide bond
Estrogen
Thyroid hormone

ABSTRACT

Earlier studies showed that protein disulfide isomerase (PDI), a well-known protein folding catalyst, can bind estrogens. Whether other PDI homologs can also bind estrogens, and if so, what are the biological functions of this unique property are not known at present and thus are the subjects of our present investigation. Here we report that, of the six representative PDI homologs examined (human PDI, PDIp, ERp57, ERp72, PDIA6 and rat PDIr), only the human pancreas-specific PDI homolog (PDIp) had a similar binding affinity for radiolabeled 17β-estradiol (E_2) as did PDI, with apparent K_d values of 1.5 ± 0.3 and 1.5 ± 0.2 μM, respectively. However, PDIp and PDI had distinctly different binding preference for several estrogen analogs. Moreover, we found that PDIp could serve as a high-capacity intracellular E_2-binding protein and could modulate the intracellular concentrations of E_2 in cultured mammalian cells as well as in human pancreatic tissue. The PDIp-bound E_2 in a cell could be released following a drop in the extracellular E_2 concentrations, and the released E_2 could then augment estrogen receptor-mediated transcriptional activity. Notably, the estrogen receptor α and β were also found to be expressed in rodent and human pancreatic tissues where high levels of PDIp were detected. Altogether, these data show that, in addition to its well-documented function as a protein folding catalyst, PDIp can also serve as an effective modulator of the cellular levels and biological actions of endogenous estrogens in certain target sites (such as the pancreas) where estrogen receptors and PDIp are co-present.

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1. Introduction

The endogenous estrogens exert a wide array of biological functions in various target tissues in females. The hormonal actions of estrogens are not only crucial for the maintenance of normal female physiology such as the development of reproductive organs and secondary sex characteristics, but they also contribute to the induction of tumors in certain target organs (e.g., pituitary, uterus and breast) [1–3]. Many of the well-known physiological actions of endogenous estrogens are mediated by the estrogen receptor (ER) α and β [4], which are transcription factors that can initiate the expression of target genes. In the past decade or so, enormous advances have been made in delineating ERα- and ERβ-mediated signalling pathways that mediate many of the known biological actions of endogenous estrogens in the body [4–6]. In addition, the non-genomic actions of ERs have also been suggested to play a role in mediating the estrogen-induced rapid signal transduction [7,8].

Besides the ER-mediated signaling pathways, other biological processes, such as metabolism, transport and storage of endogenous estrogens, also play important roles in modulating the actions of endogenous estrogens in target tissues. For instance, the interconversion between 17β-estradiol (E_2) and estrone catalyzed by 17β-hydroxysteroid dehydrogenase represents one of the most important mechanisms for regulation of estrogenic status in the uterus [9], as well as in other target tissues, including breast cancer [10]. Notably, our earlier study showed that the high levels of the oxidative 17β-hydroxysteroid dehydrogenase (Type II) contained in human breast cancer cells contribute to their selective insensitivity to the anticancer actions of 2-methoxy-E_2 (a nonpolar E_2 metabolite with strong growth-inhibitory activity) [11]. In addition to the estrogen-specific metabolizing enzymes, another well-documented modulator of global estrogenic status in the body is the sex hormone-binding globulin (SHBG). SHBG is a major E_2-binding protein present in blood, and it is estimated that >99% of

Abbreviations: PDI, protein disulfide isomerase; PDIp, pancreas-specific PDI; ERα and ERβ, estrogen receptor α and β, respectively; E_2, 17β-estrodial; T_3, 3,3′,5-triiodo-L-thyronine.

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0960-0760/$ – see front matter. Published by Elsevier Ltd.
doi:10.1016/j.jsbmb.2009.02.008

circulating E_2 is tightly bound to SHBG [12], which modulates the bioavailability of circulating E_2 and ultimately the estrogenic activity in various target tissues [13]. Beside, SHBG was also shown to be involved in mediating estrogen-induced membrane signaling pathways [13,14].

Protein disulfide isomerase (PDI), also known as P4HB or PDIA1, is a well-known protein folding catalyst and a molecular chaperone [15–17]. Earlier studies showed that this protein can bind $[^3H]E_2$ *in vitro* [18,19], and recently our data confirmed this property [20]. Differing from SHBG, PDI is an intracellular protein. Because this protein is present in liver as well as other tissues at very high levels, it has been suggested that PDI may function as a high-capacity intracellular estrogen-binding protein by serving as an intracellular estrogen reservoir and thus may modulate endogenous estrogen's biological functions [19]. This hypothesis was supported by our recent finding showing that PDI could effectively accumulate E_2 (a representative estrogen tested) inside mammalian cells and augment ER-mediated transcriptional activity of E_2 in cells that expressed high levels of PDI [20]. Given that the PDI family is composed of multiple homologs [16], it is of interest to determine whether other members of the PDI family also have E_2-binding activity, and if so, what are their potential biological functions in regulating the biological activity of endogenous estrogens.

The pancreas-specific PDI homolog (PDIp) was previously reported to be expressed in pancreatic acinar cells but not in islet cells or a few other tissues examined [21–24]. Here we report that, of the six representative PDI homologs examined in the present study, only PDIp has a similar E_2-binding activity as does PDI. Moreover, PDIp can effectively modulate the concentrations and hormonal activity of E_2 in cells. Since ERα and ERβ were also found to be co-expressed with PDIp in rodent and human pancreatic tissues, collectively these data suggest that PDIp may function as a modulator of the biological functions of endogenous estrogens in this organ.

2. Materials and methods

2.1. Chemicals, reagents, cell lines and tissues

3,3',5-Triiodo-L-thyronine (T_3), vitamin D_3 and 1,25-dihydroxy-vitamin D_3 [$1,25(OH)_2D_3$] were purchased from Sigma–Aldrich (St. Louis, MO). 17β-Estradiol (E_2) was purchased from Steraloids Inc. (Newport, RI). $[^3H]E_2$ (specific activity of 110 Ci/mmol) was obtained from PerkinElmer (Waltham, MA). All other chemicals and reagents used in this study were of analytical grade or higher.

Specific rabbit antibodies against a number of proteins were used in this study, and they were obtained from the following sources: PDI was from Sigma–Aldrich (catalog No. is P7372, dilution of 1:2000 for Western blotting), ERα from Santa Cruz (Santa Cruz, CA, sc-543, 1:200; sc-7207, 1:500), ERβ from Santa Cruz (sc-8974, 1:500) and Invitrogen (Carlsbad, CA, 51-7700, 1:100), GAPDH from Cell Signaling (Danvers, MA, 2118, 1:2000), and calnexin from Santa Cruz (sc-11397, 1:400). The endogenous estrogens contained in fetal bovine serum (FBS) were removed by using dextran-coated charcoal as described previously [25].

The mouse anti-PDIp antiserum (dilution of 1:2500 for Western blotting) was raised in our laboratory. Note that although the antibody against the recombinant human PDIp was raised in BALB/c mice (described in Supplementary Data section), we found that the antibody could also cross-react with the PDIp proteins present in C57BL/6J mouse pancreatic tissues (Fig. 7) but it had no reactivity with PDI or other proteins (see Figs. S1C and S2).

All cell lines used in this study were obtained from ATCC (Manassas, VA), and they included the MCF-7 and MDA-MB-435 human breast cancer cells; Mia Paca-2, Bx PC-3 and Capan-2 human pancreatic cancer cells; RIN-m5F rat pancreatic cells; HT22 mouse hippocampal cells; SK-N-SH human neuroblastoma cells; and monkey kidney cos-7 cells. These cell lines were cultured according to the instructions of the suppliers.

Pancreas tissues of Sprague–Dawley rats and C57BL/6J mice used in this study were collected from untreated control animals. Pancreatic tissues from rhesus monkeys were purchased from US Biomax (Rockville, MD, RhFTS151). Two human normal pancreatic tissue specimens were obtained from the National Disease Research Interchange (catalog No. is 0060960-13 and 0060884-14).

2.2. Construction of plasmids

The vectors carrying the cDNAs encoding each of the six PDI homologs, namely, human PDI, PDIp, ERp57, ERp72, PDIA6 and rat PDIr, were purchased from ATCC (I.M.A.G.E. numbers: *3879411*, *3162384*, *3542405*, *2819726*, *3454007* and *7323988*, respectively). Primers for each of these genes were designed with a respective pair of the restriction enzyme sites: 5′-NdeI/BamHI-3′ for PDI, 5′-NdeI/XhoI-3′ for PDIp, 5′-SalI/BamHI-3′ for ERp57, 5′-NdeI/BamHI-3′ for Erp72, 5′-NdeI/XhoI-3′ for PDIA6, and 5′-NdeI/BamHI-3′ for PDIr. Each gene was first cloned into the pGEM-T vector (Promega, Madison, WI), and then sub-cloned into the pET-19b vector (Novagen, La Jolla, CA), in which a short sequence of GTCGACGCTGCA (containing SalI site) had been inserted between NdeI and XhoI sites by using the Quickchange Mutation Kit (Stratagene, Cedar Creek, Texas) according to manufacturer's instructions. The pcDNA3.1 vector for protein expression in mammalian cells was purchased from Invitrogen, and PDIp was cloned into this vector at the sites of 5′-HindIII/XhoI-3′. PCR3.1-ERα and pGL-basic + ERE (estrogen responsive element) + E1b + luciferase reporter gene vector were gifts from Professor Carolyn Smith at Baylor College of Medicine (Houston, TX, USA).

2.3. Protein purification

The recombinant pET-19b vectors carrying each of the PDI homolog cDNAs were transformed into BL-21 (DE3) *E. coli* host cells, and the respective proteins were over-expressed after induction with IPTG (at 0.2 mM for Erp72 and at 0.1 mM for the other five PDI homologs) at 20 °C overnight. Cell pellets were dissolved in 10 mM sodium phosphate buffer (pH 7.4, 0.5 mM EDTA, 1 mM DTT, and 1 mM PMSF), and sonicated for 60 cycles (3 s sonication followed by 15 s break). The supernatants of bacterial cell lysates (after centrifugation at 13,200 rpm for 16 min at 4 °C) were incubated with Ni Sepharose High Performance (Amersham, Piscataway, NJ) at 4 °C for 1 h, and the unbound proteins were removed by washing 3 times with 20 mM sodium phosphate buffer (containing 0.5 M NaCl and 20 mM imidazole, pH 7.4). After elution with 0.5 M imidazole (0.5 M NaCl, pH 7.4), fractions containing target proteins (examined by SDS-PAGE analysis) were combined, desalted using a PD-10 column (Amersham), and concentrated in an Amicon Ultra-4 tube (Millipore, Billerica, MA) before stored at −80 °C. Protein concentration was determined using protein assay solution (Bio-Rad, Hercules, CA) with bovine serum albumin as standard.

The partially purified PDIp protein was further separated on the ÄKTA FPLC system using ion-exchange chromatography (Mono Q 4.6/100 PE, Amersham). The salt gradient elution was performed from 0 to 0.15 M NaCl (20 mM Tris–HCl, pH 7.5) in 2 column volumes and then gradually to 0.5 M NaCl in 25 column volumes followed by quickly increasing in 3 column volumes to 1.0 M NaCl. PDIp was eluted out with 0.35–0.4 M NaCl (see Figs. S1A and S1B) and the highly pure PDIp protein was used for raising polyclonal antibodies in female Balb/C mice.

2.4. [^{3}H]E_2-binding ability of purified PDI homolog proteins

A number of assays were used to assess the binding ability of PDI homolog proteins for [^{3}H]E_2. To determine the [^{3}H]E_2-binding activity of these purified proteins (at a final concentration of 0.5 μM) *in vitro*, each of the proteins was incubated with 13.2 nM [^{3}H]E_2 (0.1 μCi per tube) in 10 mM sodium phosphate buffer (pH 7.4) at room temperature for 2 h and then at 4 °C overnight. The samples (100 μL) were centrifuged briefly at 4 °C and subject to gel filtration separation on a PD-10 column (from Amersham) with 10 mM sodium phosphate buffer (pH 7.4, 0.15 M NaCl). Eluted fractions from 2.3 to 4.3 mL were collected and mixed with 3-volume scintillation cocktail (Fisher Scientific, Pittsburgh, PA) for radioactivity measurement with a Pelkin Beta Counter. For saturation experiments, different concentrations of [^{3}H]E_2 were present during the incubation, and the same assay method as described above was used. The non-specific binding was determined in the presence of 100-fold excess of cold E_2 for representative lower concentration points.

In addition to the use of the gel filtration method for separation of free and bound E_2, the charcoal adsorption method was also used. The samples were mixed with equal volume of a solution containing 1% dextran-coated charcoal plus T70 dextran (0.1%, *w*/*v*), incubated at 4 °C for 3 min, gently mixed 4 times during the incubation, and centrifuged at 16,000 × *g* (4 °C) for 3 min. The supernatant was mixed with scintillation cocktail for radioactivity determination.

2.5. [^{3}H]E_2-binding ability of PDI homolog proteins in E. coli and mammalian cells

We determined the [^{3}H]E_2-concentrating effect of *E. coli* cells that selectively over-expressed each of the PDI homology proteins as a means to assess the E_2-binding ability of these proteins in live cells. *E. coli* cells that over-expressed PDI homologs were cultured in glass tubes and then harvested in eppendorf tubes by centrifugation. After the cells were washed with LB medium once, they were re-suspended in cell-wall digestion buffer without lysozyme (30 mM Tris–HCl, 1 mM EDTA, 20% sucrose, pH 8.0) and adjusted to the same density (absorbance at 600 nm was 0.1). After that, lysozyme (at a final concentration of 0.3 mg/mL) and [^{3}H]E_2 (at increasing concentrations) were added to each tube before incubation at room temperature for 2 h and then at 4 °C overnight. The cell-wall digested *E. coli* cells were centrifuged at 4 °C (3000 × *g*, 8 min). Pellets (representing the spheroplasts) were broken down by treatment with 1% SDS and 0.2 M NaOH before mixed with 5-volume scintillation cocktail for radioactivity measurement. The supernatants (representing the periplasmic fracttion) were also subjected to radioactivity measurement.

For studying mammalian cells that over-expressed PDIp, they were incubated in DMEM medium (without FBS) in 24-well plates for 1 h, and then incubated with [^{3}H]E_2-containing DMEM medium for 2 h to allow [^{3}H]E_2 to enter the cells. After the medium was removed, cells were washed once with DMEM medium and digested with a lysis buffer (Promega, E1500) and subjected to radioactivity measurement.

2.6. Preparation of tissue lysates and mammalian cell microsomes

Animal tissues were homogenized in the 20 mM Tris–HCl buffer, pH 7.5, containing 0.15 M NaCl, 0.2% Triton X-100, plus protease inhibitors cocktail (Sigma–Aldrich), and then centrifuged at 4000 × *g* for 10 min at 4 °C. The obtained supernatants were used for Western blotting (see Fig. 7). Crude lysates from human pancreatic tissues were used for [^{3}H]E_2-binding assay after removal of PDIp by immunoprecipitation using the mouse anti-PDIp antiserum (the normal mouse serum was used as control).

To prepare microsomal fraction from cos-7 cells, cells were dissolved in 20 mM Tris–HCl buffer, pH 7.5, containing 0.15 M NaCl, plus the protease inhibitors cocktail, and were broken down by sonication. The cytosol was obtained after centrifugation of the whole cell lysates at 10,000 × *g* for 16 min at 4 °C. Microsomes were prepared by further centrifugation of the cytosolic fraction at 100,000 × *g* for 90 min at 4 °C. The whole cell lysates and microsomes were subject to Western blotting.

2.7. Transfection of mammalian cells

The plasmids pcDNA3.1, pcDNA3.1-PDIp and pCR3.1-ERα were used for the transfection of cos-7 cells. The transfection experiments were done in 24-well plates by using lipofectamine 2000 and Opti-MEM I reduced serum medium (both obtained from Invitrogen) according manufacturer's instructions. At 36 h after transfection, cells were subjected to [^{3}H]E_2-binding activity measurement (as described above), protein concentration determination, and Western blotting.

The plasmid pGL-basic + ERE + E1b + luciferase reporter gene was used for assaying the ERα-mediated transcriptional activity in MCF-7 cells. After transfection, MCF-7 cells were cultured with EMEM medium plus 10% DCC-treated FBS for one day and then cultured with the medium containing E_2 released form cos-7 cells for one day. Cell lysates were subjected to luciferase activity measurement (using a kit from Promega, E1500) and protein concentration determination.

3. Results

3.1. Characterization of estrogen-binding activity of the recombinant human PDIp protein

Earlier studies showed that rat PDI has E_2-binding activity [19]. Our recent study showed that human PDI can function as an intracellular estrogen-binding protein and can modulate estrogenic status in estrogen target cells that express high levels of PDI [20]. In the present study, we were interested in knowing whether other human PDI homologs, such as PDIp, Erp57, Erp72, PDIr and PDIA6 [15–17], also have this E_2-binding activity. We selectively expressed in *E. coli* these six representative PDI homologs (each with a catalytic CxxC motif and a non-catalytic thioredoxin-like domain), purified these proteins (Fig. 1A), and then determined their binding affinity for [^{3}H]E_2.

First, we used *E. coli* cells as a model (refer to our recent study [20]) to probe the binding ability of these PDI homologs for [^{3}H]E_2 in live *E. coli* cells. As shown in Fig. 1B, spheroplasts of *E. coli* cells that selectively expressed PDIp or PDI exhibited a significantly higher ability to bind [^{3}H]E_2 than the spheroplasts that carried an empty vector. However, *E. coli* cells that expressed any of the other PDI homologs did not show appreciable ability to accumulate [^{3}H]E_2. In agreement with this observation, the periplasmic fractions of *E. coli* cells that expressed PDIp or PDI concomitantly showed a lower radioactivity than the control cells carrying an empty vector or those cells expressing any of the other PDI homologs (data not shown). This initial observation suggested that PDIp (like PDI) in its natural form in live *E. coli* cells has the ability to bind [^{3}H]$E_{2,}$ whereas other PDI homologs do not share this biological property.

Then we examined the estrogen-binding activity of these six PDI homologs using purified proteins. Again, we found that only PDIp can bind [^{3}H]E_2 in a similar manner as PDI, whereas other homologs cannot bind [^{3}H]E_2 (Fig. 1C). In addition, when a different binding assay (charcoal adsorption) was used, the same observation was

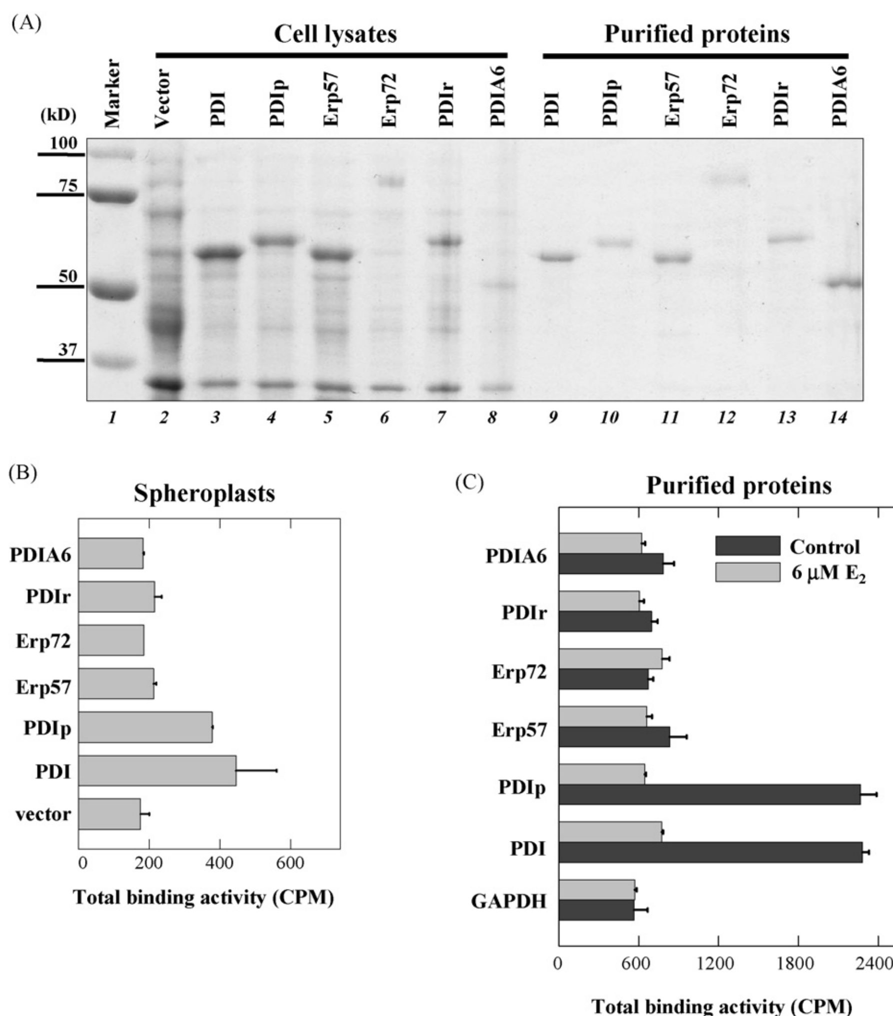

Fig. 1. Characterization of PDIp as an $[^3H]E_2$-binding protein. (A) SDS-PAGE analysis of cell lysates for PDI and five of its homologs that were over-expressed in *E. coli* cells (left part) or after each of the proteins was purified using the Nickel-affinity chromatography (*right part*). (B) The amount of radioactivity (10 nM $[^3H]E_2$) that was associated with *E. coli* spheroplasts that expressed PDI or each of the five PDI homologs. The control *E. coli* carried an empty vector (pET-19b). Cell density was adjusted to the same level by keeping the absorbance at 600 nm at 0.1 before incubation with 10 nM $[^3H]E_2$. (C) Radioactivity of $[^3H]E_2$ bound to purified PDI or each of its five homologs (at a final concentration of 0.5 μM) after incubation with 13.2 nM $[^3H]E_2$ in 10 mM sodium phosphate buffer (pH 7.4) in the absence or presence of 6 μM cold E_2. GAPDH was used as a control.

made, i.e., the $[^3H]E_2$-binding activity was only seen with PDI and PDIp (data not shown). The data from these binding analyses of purified PDI homolog proteins are in agreement with the results described above using *E. coli* cells (Fig. 1B).

Saturation binding assay showed that the recombinant human PDIp had a similar binding affinity as the recombinant human PDI (Fig. 2A and B). The nonlinear regression analysis revealed that the apparent K_d values for PDI and PDIp are 1.5 ± 0.3 μM (R^2 = 0.94) and 1.5 ± 0.2 μM (R^2 = 0.96), respectively. No significant difference in the binding affinity between the recombinant human PDI (1.5 μM, as determined in this study) and rat PDI (2.1 μM, as determined earlier [19]) was noted, which is in line with the high degree of similarity (93%) between their amino acid sequences.

Next we compared the binding preference of PDIp and PDI for several biologically important E_2 metabolites or antagonists, and significant differences were observed. As shown in Fig. 2C, whereas PDIp and PDI each had a similar binding affinity for estrone, estriol, and 4-hydroxyl-E_2, the latter obviously had a higher binding affinity for 2-methoxyl-E_2 and 2-hydroxyl-E_2. Interestingly, PDIp, unlike PDI, could not bind tamoxifen. These observations suggest a certain degree of difference in the estrogen-binding pockets of PDIp and PDI. Also, both PDIp and PDI had little binding affinity for ICI-182,780, suggesting the presence of steric hindrance caused by the long C-7α side-chain of this synthetic ER antagonist.

Since Erp57 was reported earlier to act as a receptor for the active form of vitamin D_3, $1,25(OH)_2D_3$ [26,27], we also performed the competition binding assay to determine whether D_3 and $1,25(OH)_2D_3$ can also bind to PDI and PDIp. We found that these two compounds did not have an appreciable ability to displace the binding of the radioligand $[^3H]E_2$ to PDI or PDIp, thus suggesting that they have no binding affinity for PDI and PDIp (Fig. 2C). These observations showed that the PDI family members are rather specific with respect to their ability to bind different biological ligands: while estrogens can bind to PDI/PDIp but not to Erp57, vitamin D_3 can only bind to Erp57 but not to PDI and PDIp.

Besides of the estrogen-binding activity, human PDI [28] and recombinant rat PDI [19] were previously shown to have thyroid hormone-binding activity. We observed that the binding of $[^3H]E_2$ to PDIp, like its binding to PDI, could be effectively displaced by 3,3′,5-triiodo-L-thyronine (T_3) in a concentration-dependent manner (Fig. 2D). The estimated relative binding activity (based on IC_{50} value) of PDIp for T_3 was comparable to its binding activity for PDI, approximately at 4.5 μM, which is similar to its binding activity for

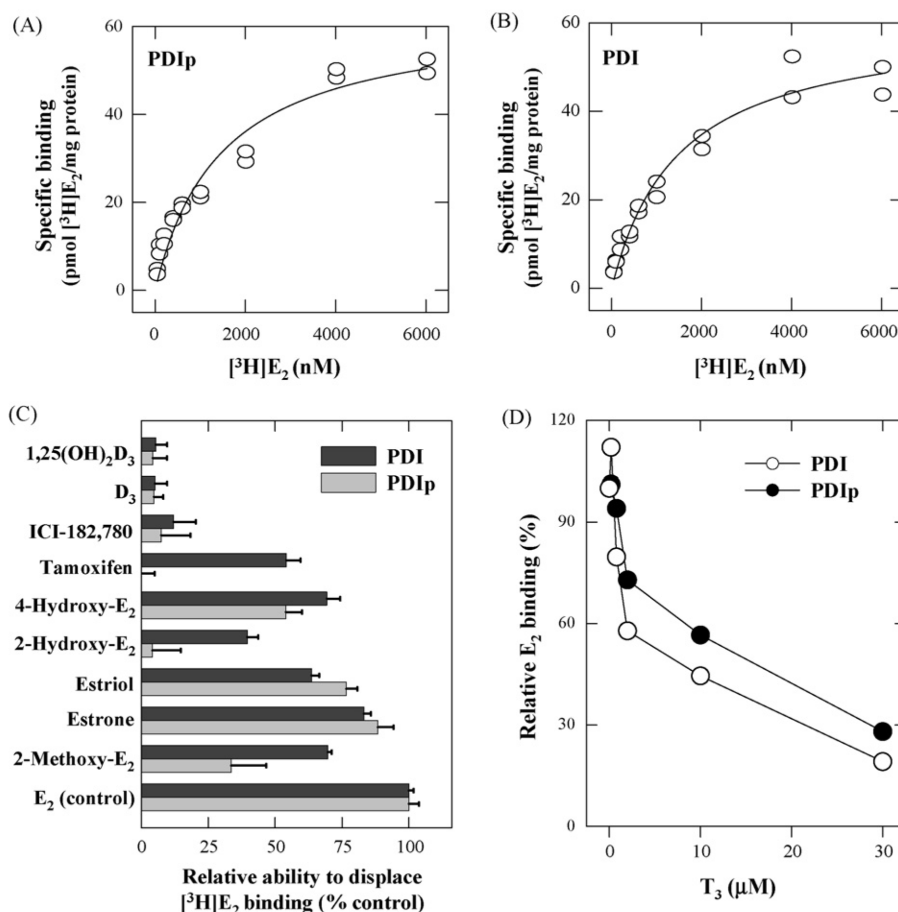

Fig. 2. Comparison of PDIp and PDI in their binding properties to estrogens The radioactivity associated with PDI (A) or PDIp (B) (at 0.5 μM final concentration) was determined in the presence of increasing concentrations of $[^3H]E_2$ (0.050–6.025 μM) in sodium phosphate buffer (10 mM, pH 7.4). The non-specific binding was determined in the presence of 100-fold excess of cold E_2 for representative lower concentration points. The curves were calculated using a nonlinear regression method (Microcal Origin software). (C) Displacement of PDIp- or PDI-bound $[^3H]E_2$ (13.2 nM) by estrogen analogs or vitamin D_3 in 10 mM sodium phosphate buffer (pH 7.4). The displacement by 6 μM cold E_2 was set as the control (100%). (D) Radioactivity associated with PDIp or PDI (at 0.5 μM final concentration) incubated with $[^3H]E_2$ (13.2 nM) in the presence of increasing concentrations of triiodothyronine (T_3) in 10 mM sodium phosphate buffer (pH 7.4). The radioactivity in the absence of T_3 was set as the control (100%).

rat PDI (4.3 μM) [19]. In addition, we found that PDIp did not have appreciable binding activity for the male sex hormone testosterone (data not shown).

3.2. The $[^3H]E_2$-binding activity of the natural PDIp protein present in human pancreas

Since the *in vitro* binding activity (data shown in Figs. 1 and 2) was based on the recombinant human PDIp selectively expressed in *E. coli* cells, next we also determined whether the PDIp present in human tissues has the same $[^3H]E_2$-binding activity. Since earlier studies showed that high levels of PDIp are expressed in human pancreas [21–24], we used this human tissue for this experiment. The pancreatic tissues were homogenized in the presence of detergent Triton X-100 that could break cellular membrane compartments (such as endoplasmic reticulum), and the tissue lysates were then incubated with mouse anti-PDIp antiserum (normal mouse serum was used as a control) to remove the PDIp protein by immunoprecipitation (Fig. 3A, upper part). Pancreatic tissue lysates after immunoprecipitation of the endogenous PDIp had approximately 40% lower specific binding activity for $[^3H]E_2$ compared to tissue lysates without removal of PDIp (Fig. 3B), indicating that PDIp present in human pancreatic tissue lysates could bind $[^3H]E_2$. Notably, the remaining $[^3H]E_2$-binding activity of the pancreatic tissue lysates after PDIp immunoprecipitation likely was attributable to the residual PDIp and also to the presence of other E_2-binding proteins (such as ERα and/or ERβ) (data are described later in Fig. 7).

3.3. Selectively expressed PDIp can modulate intracellular E_2 concentrations in live cells

Recently we have shown that PDI can help accumulate E_2 inside mammalian cells and augment ER-mediated transcriptional activity of E_2 in these cells [20]. Given that PDIp is abundantly expressed in pancreas with protein concentrations at up to 0.1% of the total cellular proteins (estimated according to data in Fig. 7), we hypothesized that PDIp might also function as a modulator of intracellular E_2 concentrations. To test this hypothesis, first we extended the observation in Fig. 1B by showing that PDIp could increase the total intracellular concentrations of $[^3H]E_2$ in *E. coli* spheroplasts when increasing concentrations of $[^3H]E_2$ were present (Fig. 4A). Consistent with this observation, a concomitant decrease in the periplasmic $[^3H]E_2$ concentrations was observed with *E. coli* cells that over-expressed PDIp (data not shown). For comparison, a similar accumulating effect on intracellular $[^3H]E_2$ levels was also observed with PDI, whereas Erp57 did not have this property.

Next we set out to determine whether the concentrating effect of PDIp on the intracellular $[^3H]E_2$ also occurs in mammalian cells. To choose appropriate cell lines for testing, we first screened a number of cell lines, including Mia Paca-2, BxPC-3, and Capan-2 human pancreatic cancer cells, RIN-m5F rodent pancreatic cancer cells, and

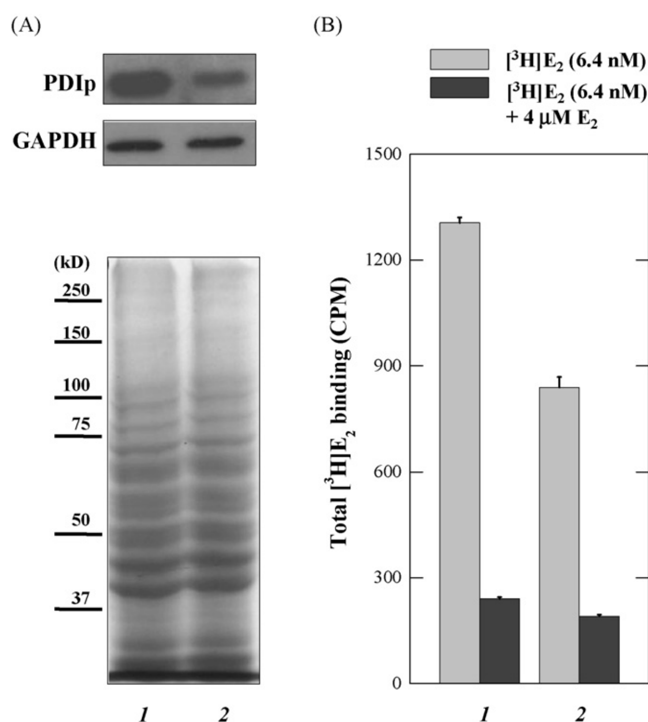

Fig. 3. $[^3H]E_2$-binding activity of PDIp prepared from human pancreas. (A) Western blotting of PDIp and GAPDH (upper part) present in the cytosol of human pancreas after immunoprecipitation. The lower part shows the Coomassie staining of pancreatic tissue lysates. Over 80% of the endogenous PDIp was removed after immunoprecipitation with mouse anti-PDIp antiserum (lane 2) compared with control mouse serum (lane 1). The protein was loaded at 2.5 μg for Western blotting and 10 μg for Coomassie staining. (B) Radioactivity of $[^3H]E_2$ bound to cytosolic proteins prepared from human pancreas. Following immunoprecipitation with anti-PDIp antiserum (column 2) or control serum (column 1), the cytosolic protein (at 0.75 mg/mL) was incubated with $[^3H]E_2$ in 20 mM Tris–HCl buffer (pH 7.5, 0.15 M NaCl, 1 mM EDTA and protease inhibitors). Free $[^3H]E_2$ and protein-bound $[^3H]E_2$ were separated by using the charcoal adsorption method. The radioactivity of $[^3H]E_2$ bound to the same concentration of GAPDH was used as background and subtracted.

several other types of cancer cell lines. Different from PDI that was found to be highly expressed in each of these cell lines (Fig. S1C), PDIp was basically not detected in any of them. The lack of expression of endogenous PDIp in these cancer cell lines prevented us from using the RNA interference to knock down PDIp in these cell lines as a study model. Alternatively, we chose to over-express PDIp in cos-7 cells, which is a commonly used mammalian cell line with a high transfection efficiency but does not express the endogenous PDIp (right part in Fig. 4B, lane 1). Following the transfection with the pcDNA3.1-PDIp plasmid, high expression of PDIp was detected in cos-7 cells (right part in Fig. 4B, lane 2). Next, we incubated cos-7 cells with $[^3H]E_2$ and measured the radioactivity associated with the cells (see Section 2). We observed a strong accumulating effect of the selectively expressed human PDIp on intracellular $[^3H]E_2$ levels (left part in Fig. 4B) when different $[^3H]E_2$ concentrations (from 5 to 100 nM) were tested, which agreed with the observations made in *E. coli* cells.

Since cos-7 cells are ER-negative [29], next we determined whether the $[^3H]E_2$-concentrating effect of PDIp as observed above (Fig. 4B) could be detected when ERα (a representative ER) was also present. To do so, we co-expressed PDIp and ERα in cos-7 cells and determined their effect on intracellular $[^3H]E_2$ levels. We observed that the intracellular $[^3H]E_2$ levels were significantly increased following the expression of ERα alone, and it was further increased when ERα and PDIp were co-expressed (Fig. 4C). This observation indicates that PDIp still can accumulate intracellular $[^3H]E_2$ in the presence of ERα.

3.4. *PDIp can increase intracellular E_2 level regardless of its localization in the cytosol or endoplasmic reticulum*

The PDI family proteins are known to be localized to the endoplasmic reticulum. However, the subcellular localization of human PDIp appears unclear at present. Alignment of human PDIp amino acid sequences available in the NCBI database (Fig. 5A) showed that the amino acid sequence of PDIp deduced from the genomic DNA sequence (CAI95586.1) theoretically should contain the full-length signal peptide, but the sequence deduced from the brain cDNA sequence (AAH00537.2) actually contains a shorter signal peptide, and PDIp identified for human pancreas (based on its cDNA sequence) does not contain any signal peptide [21]. Therefore, there is a possibility that different forms of PDIp protein may be expressed in different tissues, and the protein may have different intracellular localizations (i.e., cytosol *vs.* endoplasmic reticulum). With this possibility in mind, we also compared in this study the E_2-accumulating effect of PDIp-full (with the full-length signal peptide), PDIp-brain (with a short signal peptide), and PDIp-pancreas (without a signal peptide). The protein sequences of these three forms of PDIp are shown in Fig. 5A. We found that after the cos-7 cells were transfected with a corresponding expression plasmid for PDIp-full, PDIp-brain or PDIp-pancreas protein, each of these proteins was expressed when the whole cell lysates were analyzed using Western blotting (Fig. 5B). When the microsomal fractions prepared from the over-expressed cos-7 cells were analyzed (which also included endoplasmic reticulum membranes), much higher levels of PDIp-full and PDIp-brain proteins were detected whereas the level of PDIp-pancreas was very low (the lower part of Fig. 5B), suggesting that the former two are located in the endoplasmic reticulum whereas PDIp-pancreas is in the cytosol.

Next we also determined the accumulation of intracellular $[^3H]E_2$ in these over-expressed cos-7 cells. As shown Fig. 5C, the presence of each of the PDIp proteins accumulated more intracellular $[^3H]E_2$ than the control cells. The higher $[^3H]E_2$-accumulating effect of PDIp-full and PDIp-brain than PDIp-pancreas (Fig. 5C) was apparently due to their relative higher expression levels than the latter (compare lanes 3–6 *vs.* lane 2, using calnexin as a reference, Fig. 5B, upper part).

3.5. *Intracellular PDIp-bound E_2 can be released to augment ERα-mediated transcriptional activity*

Since PDIp can selectively accumulate E_2 in live cells in culture, next we sought to determine whether the intracellular PDIp-bound E_2 could be released upon a drop in E_2 levels and whether the released E_2 could augment its biological activity. The experimental design was summarized in Fig. 6A. Briefly, PDIp was over-expressed in ER-negative cos-7 cells (cultured in 24-well plates) by transfecting the cells with the pcDNA3.1-PDIp plasmid. Thirty-six hours later, cells were incubated with 20 nM E_2 in 0.3 mL DMEM medium (without FBS) for 2 h to allow E_2 to enter the cells. Then the E_2-containing medium was removed and cells were washed once with 1 mL EMEM medium. The cells were then incubated with 0.5 mL medium of EMEM + 10% DCC-treated FBS (E_2-free) for 2 h to release the intracellular PDIp-bound E_2 into the medium. The medium containing the released E_2 was used to culture MCF-7 cells (an ER-positive breast cancer line), and these cells were pre-transfected with a vector containing an estrogen responsive element (ERE)-driven luciferase reporter gene that enabled us to assay the ERα-mediated transcriptional activity in these cells.

As shown in Fig. 6B, the medium collected from cos-7 cells pre-incubated with 20 nM E_2 induced ERα-mediated transcriptional activity in MCF-7 cells. This effect was enhanced when MCF-7 cells were cultured by using the medium collected from PDIp-overexpressing cos-7 cells, reflecting higher E_2 concentra-

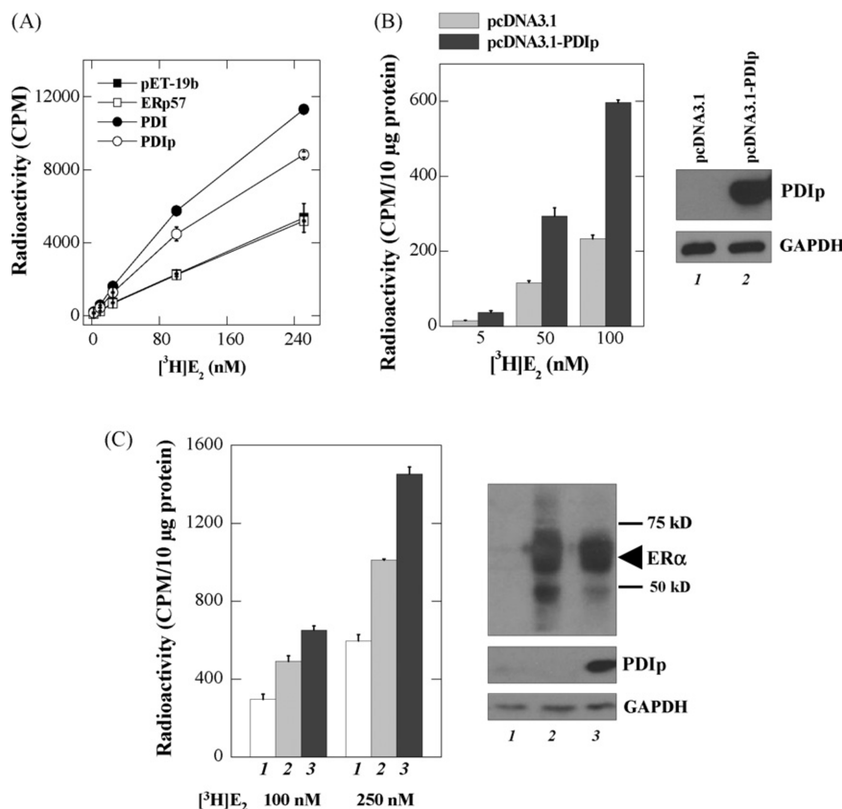

Fig. 4. PDIp modulates intracellular E_2 concentrations in *E. coli* and mammalian cells. (A) The amount of total radioactivity ($[^3H]E_2$) associated with *E. coli* spheroplasts that carried an empty vector (pET-19b) or expressed PDI, PDIp or Erp57 in the presence of increasing concentrations of $[^3H]E_2$. Cell density was adjusted to be at the same level (the absorbance at 600 nm was 0.1) before incubation with $[^3H]E_2$. (B) *Left part*: Total radioactivity associated with cos-7 cells. Cells were transfected with the pcDNA3.1 or pcDNA3.1-PDIp plasmid for 36 h, and then incubated with increasing concentrations of $[^3H]E_2$ (5, 50 and 100 nM) at 37 °C for 2 h before assaying the radioactivity content. *Right part*: Western blotting of PDIp over-expressed in cos-7 cells. Total cellular proteins were loaded at 5 µg for each well. (C) *Left part*: Total radioactivity associated with cos-7 cells. Cells were transfected with an empty pcDNA3.1 plasmid (column 1), pCR3.1-ERα + pcDNA3.1 (column 2), or pCR3.1-ERα + pcDNA3.1-PDIp (column 3) for 36 h, and then incubated with 100 or 250 nM $[^3H]E_2$ at 37 °C for 2 h before assaying the radioactivity. *Right part*: Western blotting of ERα and PDIp over-expressed in cos-7 cells. Total cellular proteins were loaded at 5 µg.

tions in the medium. Assuming that the cos-7 cells overexpressing PDIp accumulate more intracellular E_2 than control cos-7 cells (as observed in Fig. 4B), the former were expected to release more E_2 than the latter when they were incubated with E_2-free medium. Notably, the medium volume for releasing PDIp-bound E_2 in this experiment was over 200 times larger than the volume of the cos-7 cells pellet, and accordingly, there was >200-fold dilution of the intracellular E_2. Since the released E_2 after >200-fold dilution still elicited a detectable increase in ERα-mediated luciferase gene transcription, this observation suggests that the PDIp-rich cells have a rather high E_2-binding capacity.

3.6. Detection of ERα and ERβ expression in pancreatic tissues

PDIp is known to be highly expressed in the pancreas of human [21,24], sheep and rat [22], and a recent study showed that it is also expressed in mouse stomach [30]. To further probe the potential role of PDIp in modulating estrogen actions, we analyzed PDIp and also ERα and ERβ protein levels in pancreatic tissues of human (lane 1), rhesus monkey (lane 2), rat (lane 3), and mouse (lane 5), and also in mouse stomach (lane 4). As shown in Fig. 7, Western blotting showed that PDIp was highly expressed in the pancreas of all species (lanes 1–3, 5) and was also abundantly expressed in mouse stomach (lane 4). ERα (55 kD), a functional isoform of ERα [8,31], was expressed in all pancreatic tissues examined and also in mouse stomach. In comparison, the 66-kD ERα (the regular form of ERα) was found to be only expressed in mouse pancreas (lane 5) but barely detectable in human pancreas (lane 1), but not detected in the pancreas of rat and rhesus monkey (lanes 2 and 4). This pattern of expression of ERα was confirmed with another antibody against the N-terminal domain of ERα (data not shown). For ERβ (53 kD), it was detected in all tissues examined (lanes 1–5, Fig. 7), and this observation was also confirmed by using another antibody against the N-terminal domain of ERβ (data not shown). The physiological importance of the species specificity of the 66-kD ERα isoform that was found to be highly expressed in mouse pancreas merits further study.

4. Discussion

The results of our present study showed that PDIp, a pancreas-specific PDI homolog, has a similar binding activity for E_2 as does PDI (Figs. 1 and 2), and it can function as an effective intracellular E_2-binding protein and can modulate the intracellular levels of E_2 in live cells (Figs. 4B and C and 5C). This finding suggests a new biological function of PDIp *in vivo* beyond as a protein-folding catalyst, and it also aids in our understanding of its modulation of estrogen's biological functions in the pancreas, where detectable levels of ERα and ERβ expression were observed (Fig. 7).

Our finding that PDIp can selectively accumulate E_2 in cultured cells suggests that PDIp may be able to accumulate E_2 in target tissues (like pancreas) that express high levels of this protein. This possibility is supported by the observation that the endogenous PDIp present in human pancreas can accumulate E_2, which accounts for ∼40% of total E_2 bound to various cellular proteins

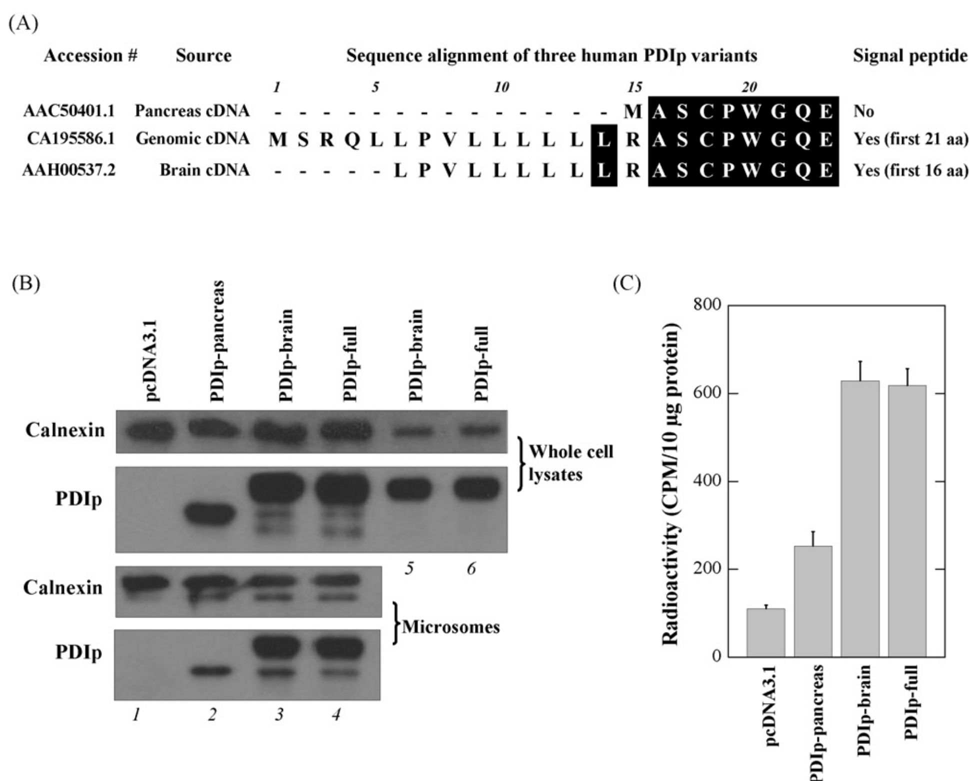

Fig. 5. PDIp accumulates E_2 in endoplasmic reticulum of mammalian cells. (A) Comparison of the N-terminal sequences of three human PDIp proteins. The protein sequences (presently available in the NCBI database) were deduced from their corresponding cDNA or genomic DNA sequences obtained from different tissues. The signal peptide analysis was done online by using the SignalP 3.0 Server (www.cbs.dtu.dk/services/SignalP-3.0/). (B) Western blotting of the relative levels of three PDIp variants in whole cell lysates and microsomal fractions prepared from cos-7 cells. Total cellular proteins were loaded at 5 μg for lanes 1–4 and at 1.7 μg for lanes 5 and 6. Calnexin, an endoplasmic reticulum membrane-bound protein, was used as a marker for the microsomal fraction. (C) Total radioactivity associated with cos-7 cells. Cells were transfected with the recombinant pcDNA3.1 plasmids for 36 h to selectively over-express three PDIp variants as described in panel A, and then incubated with $[^3H]E_2$ (50 nM) at 37 °C for 2 h before assaying the radioactivity. Here PDIp-pancreas, PDIp-brain and PDIp-full represent the AAC50401.1, AAH00537.2, and CAI95586.1 variant, respectively.

(Fig. 3B). Moreover, the PDIp-bound intracellular E_2 can be released upon a drop in the E_2 levels in the extracellular compartment and the released E_2 can augment ERα-mediated transcriptional activity (Fig. 6B). Based on these observations, it is suggested that the PDIp-bound E_2 in pancreas may help buffer the oscillations of the levels and biological activity of endogenous estrogens in the pancreas. Since PDIp expression was also detected in some other tissues (such as stomach and uterus; shown in Fig. S2), it is likely that the estrogen-accumulating effect of PDIp may also be present in these tissues.

There is an emerging body of evidence suggesting that estrogens are associated with certain physiological functions of the pancreas.

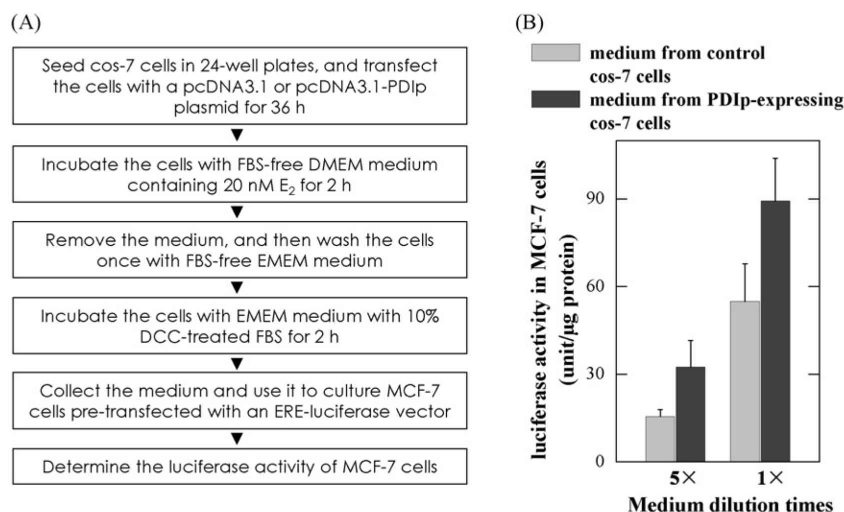

Fig. 6. Intracellular PDIp-bound E_2 can be released to augment ERα-mediated transcriptional activity. (A) A scheme depicting the experimental design. (B) Transcriptional activity of ERα in MCF-7 cells transfected with an ERE-luciferase vector. The MCF-7 cells were cultured in the medium containing E_2 that was released from cos-7 cells with or without PDIp overexpression. Luciferase activity was normalized according to the total protein concentration. The released E_2 from cos-7 cells was tested in MCF-7 cells at two dilutions (1× and 5×).

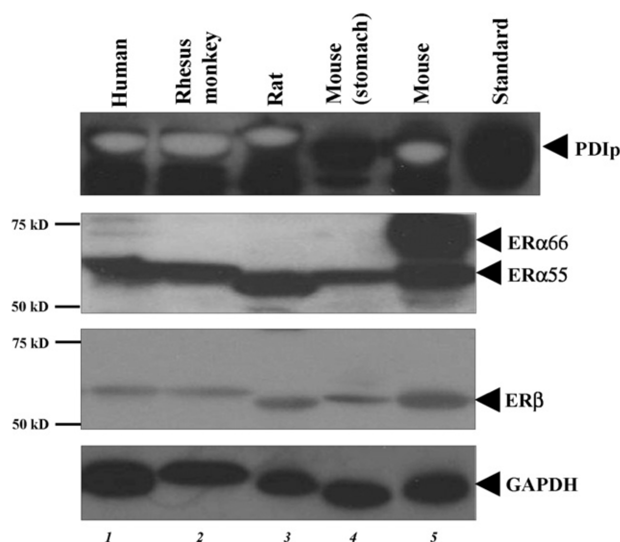

Fig. 7. Co-expression of PDIp with ERα and ERβ in the pancreas of human, rhesus monkey, rat and mouse. These tissues were homogenized in 20 mM Tris–HCl (pH 7.5, 0.15 M NaCl, 0.3% Triton X-100, 1 mM EDTA and protease inhibitors) and centrifuged at 13,000 rpm for 16 min at 4 °C. The protein concentration was adjusted to 1 mg/mL. PDIp, GAPDH, ERα and ERβ were detected by Western blotting. Mouse stomach was also analyzed (lane 4). ERα and ERβ were detected by using antibodies raised against the synthetic C-terminus peptides of human ERα and ERβ, respectively. The white bands for PDIp reflect the presence of too high levels of PDIp protein which caused over-exposure of the x-films. Total proteins were loaded at 10 μg in each well for the detection of GAPDH, ERα and ERβ, whereas 2.5 μg for detection of PDIp. Purified recombinant PDIp was loaded at 5 ng for estimation of the amount of PDIp present in each tissue.

It was observed earlier that estrogens can be directly biosynthesized in canine and human pancreas [32,33] and they can affect pancreatic zymogen production in rats [34,35]. In line with this observation, ERα and/or ERβ were found to be expressed in pancreas in our present study (Fig. 7) and also in studies by others [36,37]. Therefore, PDIp, as an intracellular estrogen-binding protein, may affect the physiological functions of estrogens in pancreas. In addition, estrogenic status has been suggested to be negatively associated with the progression of certain pancreatic cancers [37–42], which have a higher prevalence in males than in females based on both human and animal studies [38,43–45]. Given that PDIp is expressed in pancreas at very high levels, our study may provide an important missing link for the long-standing clinical speculation that estrogenic status and actions in pancreas are one of the protective factors in the development of pancreatic cancer in humans [39]. Also, it is of interest to suggest that an unknown E_2-binding protein that was detected earlier in rat pancreatic acinar cells [46,47] most likely is PDIp in light of the observations made in our present study.

In addition, we also observed that PDIp can bind a number of endogenous E_2 metabolites (Fig. 2C). This observation appears to be in line with an earlier study showing that the peptide binding activity of PDIp was inhibited by various estrogen analogs [48]. Some of these E_2 metabolites not only have important biological functions in the classic ER-mediated signaling pathways [2], but they can also exert other unique biological functions that are not shared by their parent hormone E_2. For instance, 2-methoxy-E_2 has strong apoptotic and antiangiogenic activities [49,50]; 4-hydroxy-E_2 has unique biological activity and may have its specific intracellular binding protein [51,52]; and estriol has a uniquely strong immunosuppressive effect [53]. The binding activity of PDIp for these bioactive estrogen metabolites may favorably increase their levels in the pancreas, and thus may enhance their biological actions in this organ. Interestingly, the results of our present study showed that PDIp has no binding affinity for tamoxifen (a widely used ER modulator for breast cancer treatment), which is very different from PDI. This observation may be explained according to a previous study showing that PDIp could not bind ligands with no hydroxyl group [48], since tamoxifen also does not have any hydroxyl group. It appears that the formation of hydrogen bond(s) between the hydroxyl group(s) of a ligand and PDIp is crucial for the binding interactions. PDIp, like PDI, also does not have significant binding activity for ICI-182,780 (a pure ER antagonist that has hydroxyl groups), and this observation may suggest that the binding pockets of PDIp and PDI cannot accommodate the long C-7α side-chain of this antiestrogen. Computational molecular modeling and mutagenesis studies are currently underway in our laboratory to characterize the estrogen-binding pockets of PDI and PDIp, which are expected to provide structural information on their differential binding affinity for various ligands.

In summary, of the six representative PDI homologs examined in the present study, only the pancreas-specific PDI homolog (PDIp) was found to have a similar E_2-binding activity as does PDI. Moreover, PDIp can effectively modulate the concentrations and hormonal activity of E_2 in cells. Since ERα and ERβ were found to be co-expressed with PDIp in rodent and human pancreatic tissues, these data suggest that PDIp can also serve as an effective modulator of the intracellular levels and biological actions of endogenous estrogens in certain target sites (such as the pancreas) where the ERs and PDIp are co-present.

Acknowledgements

We would like to thank Dr. Jian Ding for assistance with the production of the mouse anti-PDIp antiserum, Ms. Pan Wang for mammalian cell culture, and the NDRI for supplying human pancreatic tissues. We appreciate Professor Carolyn Smith at Baylor College of Medicine for the gift of PCR3.1-ERα and pGL-basic-ERE-luciferase plasmids. This study was supported, in part, by a grant from the NIH (CA97109). Some of the analytical and imaging instruments employed in this study are part of the COBRE core facility that is supported by the NIH Grant P20RR021940 from the National Center for Research Resources.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jsbmb.2009.02.008.

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