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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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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 subject 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 17 β -estradiol (E₂) as did PDI, with apparent K_d values of 1.5 ± 0.3 and 1.5 ± 0.2 μ M, 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 E₂-binding protein and could modulate the intracellular concentrations of E₂ in cultured mammalian cells as well as in human pancreatic tissue. The PDIp-bound E₂ in a cell could be released following a drop in the extracellular E₂ concentrations, and the released E₂ could then augment estrogen receptor-mediated transcriptional activity. Notably, the estrogen receptors 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.

Keywords

PDI; PDIp; estrogen receptor; SHBG; pancreas; disulfide bond; estrogen; thyroid hormone

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.,

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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 ER α - and ER β -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 17 β -estradiol (E₂) and estrone catalyzed by 17 β -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 17 β -hydroxysteroid dehydrogenase (Type II) contained in human breast cancer cells contribute to their selective insensitivity to the anticancer actions of 2-methoxy-E₂ (a nonpolar E₂ 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 E₂-binding protein present in blood, and it is estimated that >99% of circulating E₂ is tightly bound to SHBG [12], which modulates the bioavailability of circulating E₂ and ultimately estrogenic activity in various target tissues [13]. Besides, 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]E₂ *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 E₂ (a representative estrogen tested) inside mammalian cells and augment ER-mediated transcriptional activity of E₂ 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 E₂-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 E₂-binding activity as does PDI. Moreover, PDIp can effectively modulate the concentrations and hormonal activity of E₂ in cells. Since ER α and ER β 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

Triiodothyronine (T₃), vitamin D₃ and 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] were purchased from Sigma-Aldrich (St. Louis, MO). 17 β -Estradiol (E₂) was purchased from Steraloids Inc. (Newport, RI). [³H]E₂ (specific activity of 110 Ci/mmol) was obtained from

Perkin Elmer (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), ER α from Santa Cruz (Santa Cruz, CA, sc-543, 1:200; sc-7207, 1:500), ER β 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 the **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 **Fig. S1C, Fig. 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 supplier's instructions.

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-ER α 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 three 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 **Fig. S1A, S1B**) and the highly-pure PDIp protein was used for raising polyclonal antibodies in female Balb/C mice.

2.4. [³H]E₂-binding ability of purified PDI homolog proteins

A number of assays were used to assess the binding ability of PDI homolog proteins for [³H]E₂. To determine the [³H]E₂-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 [³H]E₂ (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 desalting 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 [³H]E₂ were present during the incubation, and the same assay method as described above was used. The nonspecific binding was determined in the presence of 100-fold excess of cold E₂ for representative lower concentration points.

In addition to the use of the desalting method for separation of free and bound E₂, 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 four 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. [³H]E₂-binding ability of PDI homolog proteins in *E. coli* and mammalian cells

We determined the [³H]E₂-concentrating effect of *E. coli* cells that selectively over-expressed each of the PDI homology proteins as a means to assess the E₂-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 washed with LB medium once, the cells 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 [³H]E₂ (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 fraction) were also subjected to radioactivity measurement.

For studying mammalian cells that over-expressed PDIp, they were incubated in DMEM medium (without FBS) for 1 h, and then incubated with [^3H]E $_2$ -containing DMEM medium for 2 h to allow [^3H]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 4,000 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 [^3H]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 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-ER α were used for assaying the [^3H]E $_2$ -binding in cos-7 cells. 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 [^3H]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 ER α -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 from 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, 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 [^3H]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 [^3H]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 [^3H]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 [^3H]E₂. 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 (likePDI) in its natural form has the ability to bind [^3H]E₂, 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 [^3H]E₂ in a similar way as PDI, whereas other homologs can not bind [^3H]E₂ (Fig. 1C). In addition, when a different binding assay (charcoal adsorption) was used, the same observation was made, *i.e.*, the [^3H]E₂-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, 2B). The nonlinear regression analysis revealed that the apparent K_d values for PDI and PDIp are $1.5 \pm 0.3 \mu\text{M}$ ($R^2 = 0.94$) and $1.5 \pm 0.2 \mu\text{M}$ ($R^2 = 0.96$), respectively. No significant difference in the binding affinity between the recombinant human PDI ($1.5 \mu\text{M}$, as determined in this study) and rat PDI ($2.1 \mu\text{M}$, 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 E₂ 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-E₂, the latter obviously had a higher binding affinity for 2-methoxyl-E₂ and 2-hydroxyl-E₂. Interestingly, PDIp, unlike PDI, could not bind tamoxifen. These observations suggest a certain degree of difference in the ligand-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-7 α side-chain of this synthetic ER antagonist.

Since Erp57 was reported earlier to act as a receptor for the active form of vitamin D₃, 1,25(OH)₂D₃ [26,27], we also performed the competition binding assay to determine whether D₃ and 1,25(OH)₂D₃ 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]E₂ 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 D₃ 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]E₂ to PDIp, like its binding to PDI, could be effectively displaced by 3,3',5-triiodo-L-thyronine (T₃) in a concentration-dependent manner (Fig. 2D). The estimated relative binding activity (based on IC_{50} value) of PDIp for T₃ was comparable to its binding activity for PDI, approximately at $4.5 \mu\text{M}$, which is similar to its binding activity for rat PDI ($4.3 \mu\text{M}$) [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]E₂-binding activity of the natural PDIp protein present in human pancreas

Since the *in-vitro* binding activity (data shown in Fig. 1, 2) was based on the recombinant human PDIp selectively-expressed in *E. coli* cells, next we also determined whether the PDIp

present in human pancreas 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 human pancreas for this purpose. 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). 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 ER α and/or ER β) (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 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 Materials and Methods). 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 ER α (a representative ER) was also present. To do so, we co-expressed PDIp and ER α 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 ER α alone, and it was further increased when ER α and PDIp were co-expressed (Fig. 4C). This observation indicates that PDIp still can accumulate intracellular [^3H]E $_2$ in the presence of ER α .

3.4. PDIp can increase intracellular E₂ level regardless of its localization in the cytosol or endoplasmic reticulum

The PDIp family proteins are known to be associated with 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 E₂-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]E₂ in these over-expressed cos-7 cells. As shown Fig. 5C, the presence of each of the PDIp proteins accumulated more intracellular [³H]E₂ than the control cells. The higher [³H]E₂-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, 4, 5, 6 *vs* lane 2, using calnexin as a reference, Fig. 5B, upper part).

3.5. Intracellular PDIp-bound E₂ can be released to augment ER α -mediated transcriptional activity

Since PDIp can selectively accumulate E₂ in live cells in culture, next we sought to determine whether the intracellular PDIp-bound E₂ could be released upon a drop in E₂ levels and whether the released E₂ 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 h later, cells were incubated with 20 nM E₂ in 0.3 mL DMEM medium (without FBS) for 2 h to allow E₂ to enter the cells. Then the E₂-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 (E₂-free) for 2 h to release the intracellular PDIp-bound E₂ into the medium. The medium containing the released E₂ 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 ER α -mediated transcriptional activity in these cells.

As shown in Fig. 6B, the medium collected from cos-7 cells pre-incubated with 20 nM E₂ induced ER α -mediated transcriptional activity in MCF-7 cells. This effect was enhanced when MCF-7 cells were cultured by using medium collected from PDIp-overexpressing cos-7 cells, reflecting higher E₂ concentrations in the medium. Assuming that the cos-7 cells overexpressing PDIp accumulate more intracellular E₂ than control cos-7 cells (as observed in Fig. 4B), the former were expected to release more E₂ than the latter when they were

incubated with E₂-free medium. Notably, the medium volume for releasing PDIp-bound E₂ 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 E₂. Since the released E₂ after >200-fold dilution still showed a detectable increase in ER α -mediated luciferase gene transcription, this observation suggests that the PDIp-rich cells have a rather high E₂-binding capacity.

3.6. Detection of ER α and ER β 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 ER α and ER β 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, 2, 3, 5) and was also abundantly expressed in mouse stomach (lane 4). ER α (55 kD), a functional isoform of ER α [8,31], was expressed in all pancreatic tissues examined and also in mouse stomach. In comparison, the 66-kD ER α (the regular form of ER α) 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 ER α was confirmed with another antibody against the N-terminal domain of ER α (data not shown). For ER β (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 ER β (data not shown). The physiological importance of the species specificity of the 66-kD ER α 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 E₂ as does PDI (Fig. 1, 2), and it can function as an effective intracellular E₂-binding protein and can modulate the intracellular levels of E₂ in live cells (Fig. 4B, 4C, 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 expression levels of ER α and ER β were observed (Fig. 7).

Our finding that PDIp can selectively accumulate E₂ in cultured cells suggests that that PDIp may be able to accumulate E₂ 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 E₂, which accounts for ~40% of total E₂ bound to various cellular proteins (Fig. 3B). Moreover, the PDIp-bound intracellular E₂ can be released upon a drop in the E₂ levels in the extracellular compartment and the released E₂ can augment ER α -mediated transcriptional activity (Fig. 6B). Based on these observations, it is suggested that the PDIp-bound E₂ 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. 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, ER α and/or ER β 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 E₂-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 E₂ 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 E₂ 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 E₂. For instance, 2-methoxy-E₂ has strong apoptotic and antiangiogenic activities [49,50]; 4-hydroxy-E₂ 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 which showed 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-7 α side-chain of this antiestrogen. Computational molecular modeling and mutagenesis studies are currently underway in our laboratory to characterize the ligand-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 E₂-binding activity as has PDI. Moreover, PDIp can effectively modulate the concentrations and hormonal activity of E₂ in cells. Since ER α and ER β 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.

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Abbreviations

PDI
protein disulfide isomerase

PDIp

pancreas-specific PDI

ER α and ER β estrogen receptor α and β respectively**E₂**17 β -estradiol**T₃**

3,3',5-triiodo-L-thyronine

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FIGURE 1. Characterization of PDIp as an [3 H] 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 [3 H] 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 [3 H] E_2 . **C.** Radioactivity of [3 H] E_2 bound to purified PDI or each of its five homologs (at a final concentration of 0.5 μ M) after incubation with 13.2 nM [3 H] E_2 in 10 mM sodium phosphate buffer (pH 7.4) in the absence or presence of 6 μ M cold E_2 . GAPDH was used as a control.

FIGURE 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]E₂ (0.050 to 6.025 μM) in sodium phosphate buffer (10 mM, pH 7.4). The nonspecific binding was determined in the presence of 100-fold excess of cold E₂ 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]E₂ (13.2 nM) by estrogen analogs or vitamin D₃ in 10 mM sodium phosphate buffer (pH 7.4). The displacement by 6 μM cold E₂ was set as the control (100%). D. Radioactivity associated with PDIp or PDI (at 0.5 μM final concentration) incubated with [³H]E₂ (13.2 nM) in the presence of increasing concentrations of triiodothyronine (T₃) in 10 mM sodium phosphate buffer (pH 7.4). The radioactivity in the absence of T₃ was set as the control (100%).

FIGURE 3. $[^3\text{H}]\text{E}_2$ -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 $[^3\text{H}]\text{E}_2$ 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 $[^3\text{H}]\text{E}_2$ in 20 mM Tris-HCl buffer (pH 7.5, 0.15 M NaCl, 1 mM EDTA and protease inhibitors). Free $[^3\text{H}]\text{E}_2$ and protein-bound $[^3\text{H}]\text{E}_2$ were separated by using the charcoal adsorption method. The radioactivity of $[^3\text{H}]\text{E}_2$ bound to the same concentration of GAPDH was used as background and subtracted.

FIGURE 4. PDIp modulates intracellular E_2 concentrations in *E. coli* and mammalian cells

A The amount of total radioactivity ($[^3\text{H}]\text{E}_2$) 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 $[^3\text{H}]\text{E}_2$. Cell density was adjusted to be at the same level (the absorbance at 600 nm was 0.1) before incubation with $[^3\text{H}]\text{E}_2$. **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 $[^3\text{H}]\text{E}_2$ (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-ER α + pcDNA3.1 (column 2), or pCR3.1-ER α + 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 ER α and PDip over-expressed in cos-7 cells. Total cellular proteins were loaded at 5 μg .

FIGURE 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 [3 H] 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.

FIGURE 6. Intracellular PDIP-bound E₂ can be released to augment ER α -mediated transcriptional activity

A A scheme depicting the experimental design **B**. Transcriptional activity of ER α in MCF-7 cells transfected with an ERE-luciferase vector. The MCF-7 cells were cultured in the medium containing E₂ that was released from cos-7 cells with or without PDIP overexpression. Luciferase activity was normalized according to the total protein concentration. The released E₂ from cos-7 cells was tested in MCF-7 cells at two dilutions (1 $\times$ and 5 $\times$).

FIGURE 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.