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ERp57 binds competitively to protein disulfide isomerase and calreticulin ☆

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Abstract

In this study, we screened for protein disulfide isomerase (PDI)-binding proteins in bovine liver microsomes under strict salt concentrations, using affinity column chromatography. One main band observed using SDS–PAGE was identified as ERp57 (one of the PDI family proteins) by LC-MS/MS analysis. The K_D value of PDI binding to ERp57 was calculated as 5.46×10^{-6} M with the BIACORE system. The interactions between PDI and ERp57 occurred specifically at their a and b domains, respectively. Interestingly, low concentrations of ERp57 enhanced the chaperone activity of PDI, while high concentrations interfered with chaperone activity. On the other hand, ERp57 did not affect the isomerase activity of PDI. Additionally, following pre-incubation of ERp57 with calreticulin (CRT), decreased interactions were observed between ERp57 and PDI, and vice versa. Based on the data, we propose that once ERp57 binds to PDI or CRT, the resultant complex inhibits further interactions. Therefore, ERp57 selectively forms a protein-folding complex with PDI or CRT in ER.

Keywords: Protein disulfide isomerase; ERp57; Calreticulin; Chaperone; BIACORE system

Protein disulfide isomerase (PDI), one of the major enzymes that assist protein folding, catalyzes the formation, reduction, and isomerization of protein disulfide bonds in the endoplasmic reticulum (ER) [1]. PDI contains two thioredoxin-like motifs (CXXC) in two separate domains (a and a′), and an ER retention signal, KDEL, at the C-terminus [2]. PDI displays both chaperone and anti-chaperone activities [3], and is possibly involved in the quality control system whereby misfolded proteins are destined for degradation in the cell [4]. Moreover, PDI functions as subunits of prolyl-4-hydroxylase [5] and microsomal triglyceride transfer protein [6]. It is evident that PDI is a multifunctional protein.

Several PDI family proteins have been identified, including ERp57 [7–10], P5 [11], PDIR [12], and ERp72 [13], that are similar to PDI in structure. PDI

☆ *Abbreviations:* CNX, calnexin; CRT, calreticulin; ER, endoplasmic reticulum; FPLC, fast protein liquid chromatography; PDI, protein disulfide isomerase; RU, resonance units.

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family proteins are characterized by two or three CXXC motifs in their primary structures. The functions of these proteins remain to be clarified in vivo. However, the role of ERp57 is relatively well characterized. Recently, PDI and ERp57 proteins were identified from nucleoli isolated from cultured human cells [14]. ERp57 contains two CGHC motifs at the active site and the ER retention signal 'QDEL' at the C-terminus [8]. The protein specifically interacts with calnexin (CNX) and calreticulin (CRT) [15,16], which are involved in the folding of newly synthesized glycoproteins, to form the correct disulfide bonds [17,18]. Calreticulin comprises N, P, and C domains. The N domain binds to monoglycosylated glycans, while the C domain contains a peptide-binding site as the chaperone region. Both b and b′ domains of ERp57 bind the P domains of CNX and CRT, respectively [19–21].

We previously reported that PDI interacts with several antibiotics, which interfere with its chaperone activity [22]. In this study, we show for the first time that PDI interacts with ERp57. Moreover, at low concentrations, ERp57 increases the chaperone activity of PDI up to approximately 1.43-fold. In conjunction with the above findings, our data show that ERp57 binds to both PDI and CRT. Binding experiments using several domains of PDI and ERp57 disclose that both a and b domains of these proteins are involved in their interactions. Moreover, the interactions between ERp57, PDI, and CRT are competitive. Here, we report that ERp57 competitively forms a protein complex with PDI or CRT and discuss the role of the complex in ER.

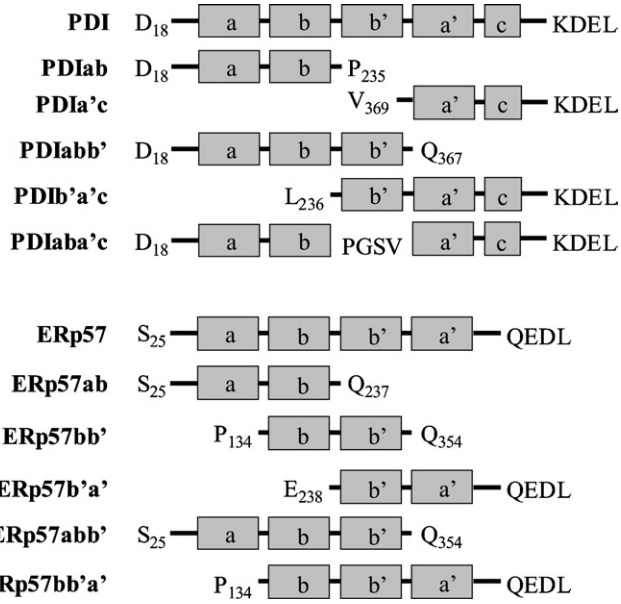


Fig. 1. The domain structures of hPDI, hERp57, and their mutants. The domains depicted as a and a′ are redox-active thioredoxin domains, b and b′ are redox-inactive thioredoxin domains, while c is a putative calcium-binding domain. The C-terminal KDEL and QDEL sequences are possible ER retention signals.

Materials and methods

Materials. HiTrap NHS-activated HP column and bovine liver were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden) and Nippon Ham (Osaka, Japan), respectively. Bovine PDI protein was purified from liver, using a method described previously [23].

Strains and plasmids. *Escherichia coli* AD494 (DE3) {Δ*ara*$^{-}$, *leu7967*, Δ*lacX74*, Δ*phoA*, *Pvu II*, *phoR*, Δ*malF3*, *F*′[*lac*$^{+}$, (*lacI*^{q}), *pro*], *trxB*::*kan*(*DE3*)} and pET15b (Novagen, Madison, WI, USA) were used to express human PDI (hPDI), human ERp57 (hERp57), their domains (Fig. 1), and human calreticulin (hCRT). Recombinant hPDI and hCRT were prepared, as described previously [24,25]. For generating hERp57, *Xho*I and *Bam*HI sites were introduced 1–6 nucleotides upstream of the 5′-terminus of the region encoding the secretary leader peptide, and 4–9 nucleotides downstream of the coding region of the ER retention signal, using 5′-CCATATGCTCGAGTCCGACGTGCTA-3′ and 5′-AGCC GGATCCTTAGAGATCCTCCTGT-3′ as the upper and lower primers, respectively. The PCR product obtained was inserted into the corresponding restriction sites of pET15b. To generate domain mutants of hPDI, *Nde*I sites were introduced 1–6 nucleotides upstream and downstream of the amino acids depicted in Fig. 1, respectively. Similarly, to prepare domain mutants of hERp57, *Xho*I and *Bam*HI sites were introduced, as described above. The appropriate fragments were cloned in the *Nde*I and *Xho*I/*Bam*HI sites of pET15b, respectively.

Preparation of microsomes from bovine liver and screening of proteins binding to bovine PDI. Bovine liver was cut into small sections and homogenized in 0.25 M sucrose with an Excel Auto Homogenizer (Nippon Seiki, Japan), followed by a Teflon homogenizer (Rikagaku Garasu Seisakujo, Japan). The homogenate was centrifuged at 13,300*g* for 10 min at 4 °C and the supernatant at 105,000*g* for 60 min at 4 °C. The resulting pellet was suspended in 0.15 M KCl buffer. After centrifugation of the suspension at 105,000*g* for 30 min at 4 °C, the pellet was suspended in buffer [0.15 M KCl, 0.05 M KH_2PO_4, and 0.05 M K_2HPO_4 (pH 7.5)]. Next, the suspension was centrifuged at 100,000*g* for 90 min at 4 °C, and the ensuing pellet was dissolved in S-TKM buffer (pH 7.5) [50 mM Tris, 25 mM KCl, 5 mM $MgCl_2$, 0.25 M sucrose, and 0.5% (v/v) Triton X-100]. The suspension was centrifuged at 100,000*g* for 90 min at 4 °C, and the supernatant obtained was used as the microsome fraction. Finally, PDI was removed from the microsome fraction using an earlier purification method [23], and the sample containing 180 mM NaCl was applied to a PDI-Sepharose affinity column. Unbound materials were thoroughly washed out with buffer [20 mM Tris–HCl (pH 7.2) containing 180 mM NaCl], and bound proteins were eluted with elution buffer [20 mM Tris–HCl (pH 7.2) containing 1 M NaCl]. To identify the proteins that bound to PDI, SDS–PAGE was performed according to the method of Laemmli [26] using 12.5% (w/v) gel. Proteins were detected with a silver staining kit (Wako, Japan).

LC-MS/MS. SDS–PAGE gel fragments containing proteins were excised and subjected to in-gel trypsin digestion, as described earlier [27]. Peptides generated were analyzed using an LC-MS/MS system [28]. The peptide mixture was separated on a fritless Mightysil-C18 (3 μm particles, Kanto Chemical, Osaka, Japan) column (0.150 mm i.d., 450 mm length), using a gradient of acetonitrile (0–40%) in 0.1% formic acid at a flow rate of 100 ml/min with a Direct nano-LC system (Nanosolution, Tokyo, Japan). Eluted peptides were sprayed directly into a quadrupole time-of-flight hybrid mass spectrometer (Q-Tof2, Micromass, Manchester, UK). MS/MS spectra were acquired by data-dependent collision-induced dissociation, and the data obtained were analyzed using MASCOT software (Matrix Science, Wyndham Place, UK) for peptide assignment [29].

Expression and purification of His-tagged hPDI, hERp57, several of their domains, and hCRT. *Escherichia coli* AD494(DE3) was trans-

formed with plasmids of the pET15b series [24,25], and transformants were grown at 3 °C in LB medium containing 100 μg/ml ampicillin with shaking. At an OD_{600nm} of 0.4–0.6, IPTG was added to a final concentration of 1 mM and incubation was continued at 30 °C for 6 h. Cells were collected by centrifugation at 6000 rpm for 10 min at 4 °C, suspended in 20 mM sodium phosphate buffer (pH 7.4), and disrupted with an ultrasonic cell disrupter. The supernatant was passed through a Minisart (Sartorius) (0.2 μm) and applied to a Ni^{2+}-chelating resin column (Amersham Pharmacia Bioscience) for purification.

Biomolecular interactions. Surface plasmon resonance experiments were performed with the BIACORE biosensor system 3000 (Biacore, Uppsala, Sweden). Specifically, hPDI, hERp57, their domains or hCRT was immobilized on the surface of CM5 sensor chip via *N*-hydroxysuccinimide and *N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide activation chemistry, according to the manufacturer's instructions. Unreacted carboxymethyl groups of a sensor chip lacking immobilized proteins were blocked with ethanolamine as a control for non-specific binding. As analytes, hPDI, hERp57, hCRT or a mixture of hPDI, hERp57, and hCRT were injected over the flow-cell at a rate of 20 μl/min at 25 °C. HBS [0.01 M Hepes, 0.15 M NaCl, 0.005% Tween 20, and 3 mM EDTA (pH 7.4)] was used as running buffer during the assay to exclude non-specific binding. Data analysis was performed using BIA evaluation ver.3.1 software.

Effects of hERp57 on hPDI activities. The isomerase activities of PDI family proteins were determined using the method of Ibbetson and Freedman [30] in which the enzyme-catalyzed reduction of disulfide bonds of insulin by GSH is coupled to the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) by NADPH and glutathione reductase. Chaperone activity measured as the level of prevention of denatured rhodanese aggregation was analyzed using the method described by Martin et al. [31]. Briefly, bovine rhodanese was denatured in buffer A (6 M guanidium–HCl, 30 mM Tris–HCl, and 1 mM dithiothreitol [pH 7.2]), and aggregation of denatured rhodanese was determined by monitoring the increase in absorbance at 320 nm.

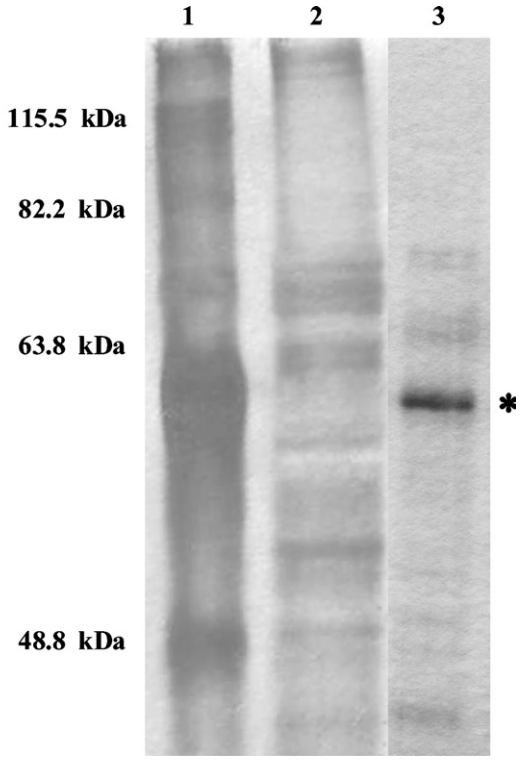


Fig. 2. Isolation of PDI-binding proteins using PDI affinity chromatography. Lane 1, marker proteins; lane 2, fraction eluted with wash buffer [20 mM Tris–HCl, pH 7.2, 180 mM NaCl]; and lane 3, fraction containing PDI-binding proteins eluted with elution buffer [20 mM Tris–HCl buffer, pH 7.2, 1 M NaCl]. The band with an asterisk (*) indicates ERp57.

Results

Specific binding of PDI to ERp57

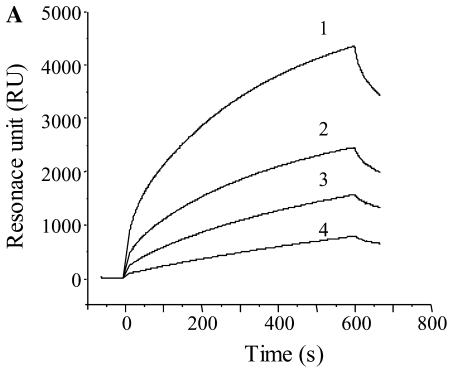
In this study, we identified PDI-binding proteins from microsomes of bovine liver using the PDI-Sepharose column in the presence of 180 mM NaCl. One main band observed with SDS–PAGE (Fig. 2) was identified as ERp57 by LC-MS/MS analysis. ERp57 is a PDI family protein that facilitates the formation of disulfide bonds in nascent glycoproteins. To determine the affinity of ERp57 against PDI, we purified recombinant human ERp57 and PDI from *E. coli*. Purified hPDI was immobilized on a CM5 sensor chip, and interactions between hPDI and hERp57 were analyzed using BIACORE 3000. Notably, hERp57 binding to and dissociation from hPDI were gradual processes (Fig. 3). The K_D value of hERp57 binding to hPDI was determined as 5.46×10^{-6} M (Fig. 3).

Domains of hPDI and hERp57 essential for interactions

To identify the domain of hPDI essential for interactions with hERp57, several domain fragments of hPDI were expressed in *E. coli* and purified as soluble proteins. We additionally generated several domain mutants of hERp57. The recombinant fragments of hPDI and hERp57 produced in this study are listed in Fig. 1. The ab, bb′a′, and b′a′ fragments of hERp57 were soluble, while bb′ and abb′ were insoluble (data not shown). Only the soluble fragments were employed for subsequent interaction analyses. The K_D values of hPDIabb′ and hPDIaba′c for binding to hERp57 were 5.97×10^{-6} and 8.95×10^{-6} M, respectively. However, affinities of hPDI-ab, hPDI-b′a′c, and hPDI-a′c for hERp57 were not detected (Table 1). These results indicate that the b′, a′, and c domains of hPDI are unnecessary for interactions between hPDI and hERp57. However, only the a and b domains of PDI are insufficient for binding to hERp57. Interestingly, the a and b domains of hERp57 are additionally essential for interactions with hPDI (Table 1).

Effects of hERp57 on the chaperone and isomerase activities of hPDI

PDI has two functions, specifically, isomerase and chaperone activities. Accordingly, we examined whether interactions with hERp57 affect the activities of hPDI. Initially, we predicted that hPDI binds to hERp57 via disulfide bond formation between particular domains



B

	K_D (M) to ERp57
PDI	5.46×10^{-6}

Fig. 3. Analysis of interactions between PDI and ERp57 by SPR. All analytes [1 (7.68 μM), 2 (3.84 μM), 3 (1.92 μM), and 4 (0.96 μM)] of ERp57 shown in the inset were injected over immobilized hPDI (A). (B) The dissociation constant was calculated using BIA evaluation 3.0.

Table 1
Interaction between hPDI and hERp57

Analyte	Dissociation constant (M)	
	Ligand	
	PDI	ERp57
PDIab	—	ND
PDIa′c	—	ND
PDIabb′	—	5.97×10^{-6}
PDIb′a′c	—	ND
PDIaba′c	—	8.95×10^{-6}
ERp57ab	3.93×10^{-6}	—
ERp57b′a′	ND	—
ERp57bb′a′	ND	—

The data analysis was performed using the BIA evaluation ver.3.1 software. ND, not detected; —, not determined.

of each protein, and that isomerase activity of hPDI is decreased. However, our data showed that hERp57 did not affect the isomerase activity of hPDI (Fig. 4A). On the other hand, low concentrations of added hERp57 increased the chaperone activity of hPDI (up to 143%) (Fig. 4B). Unexpectedly, high concentrations of ERp57 suppressed the activity (Fig. 4B). The reason for this is currently unclear. We additionally examined the interactions between hPDI-m12 and hERp57-m12 in which all the cysteine residues of the CXXC motifs were replaced with serines [24]. The resulting interactions were confirmed on the BIACORE system (data not shown). The results clearly demonstrate that cysteine residues of the CXXC motif do not participate in interactions between PDI and ERp57.

Competitive binding of hERp57 to hPDI and hCRT

The b and b′ domains of ERp57 bind to the P domains of CNX and CRT [19–21]. Moreover, the a and b domains of hPDI and hERp57 are necessary for interactions between these proteins (Table 1). In view of these results, we examined whether PDI forms a complex with ERp57 in the presence of CRT. We injected pre-incubated ERp57-CRT or ERp57-PDI as analytes onto sensor chip-immobilized PDI and CRT, respectively, using the BIACORE system. As shown in Fig. 5A, the association and dissociation of ERp57-PDI represents a typical sensorgram of a protein–protein interaction. However, the binding of ERp57-PDI was distinct from that of ERp57-CRT (Fig. 5B). The resonance unit of each pre-incubated sample was lower than that of ERp57 alone, and PDI and CRT inhibited the binding of ERp57 to each other (Fig. 5). Accordingly, we suggest that ERp57 interacts competitively with PDI and CRT, and that each complex participates in protein folding in the ER.

Discussion

PDI interacts with several proteins in the cell and sometimes functions as a subunit of proteins [5,6]. For example, a recent report shows that PDI and Bip concertedly assist in the folding of synthetic proteins in the ER [32,33], and PDI couples with ERO1, which helps the disulfide bond exchange [34,35]. In this study, we show that PDI interacts with ERp57 in buffer containing 180 mM NaCl. The K_D value of ERp57 for PDI was 5.46×10^{-6} M, as measured using the BIACORE system. Moreover, recombinant domain analyses disclosed that their a and b domains are required for these interactions. ERp57 did not affect the isomerase activity of PDI, and PDI and ERp57 interacted, even when the cysteine residues present in their CXXC motifs were replaced with serines. On the other hand, the chap-

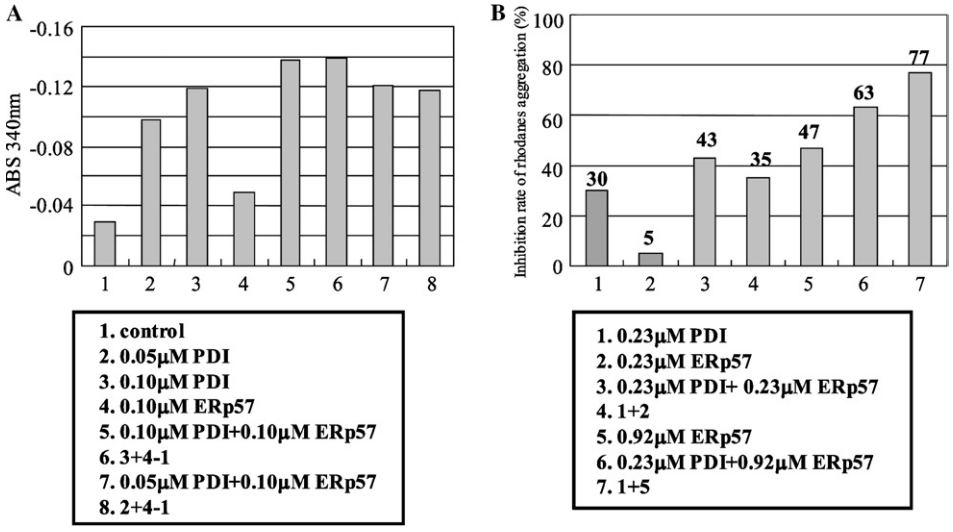


Fig. 4. Effect of ERp57 on the isomerase and chaperone activities of PDI. (A) The isomerase activities of PDI or/and ERp57 were determined according to the method of Ibbetson and Freedman [30]. Experiments done in the absence of PDI and ERp57 were used as controls. No acceleration in the reduction of disulfide bonds of insulin by PDI was observed in the presence of ERp57. (B) The inhibition rate of rhodanese aggregation after a 15 min reaction is presented. The inhibition rate was calculated by subtracting the aggregation percent of rhodanese from the spontaneous aggregation percent (100%).

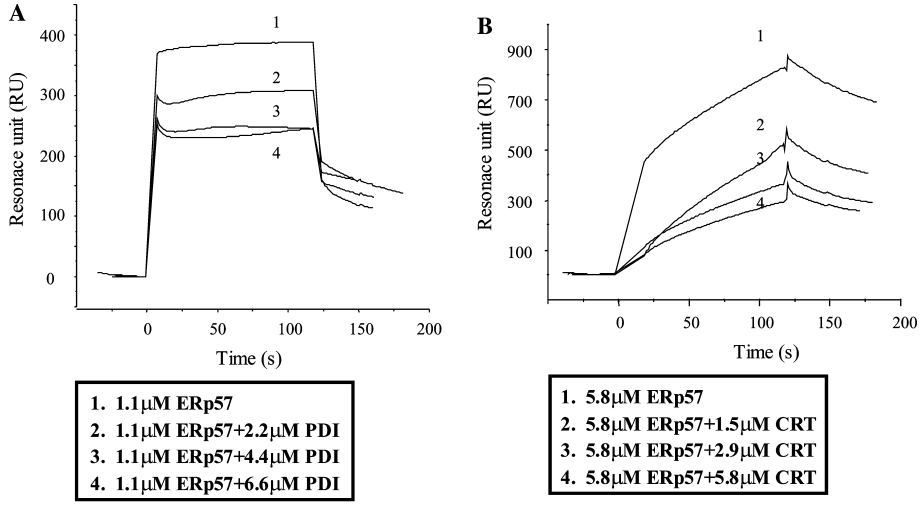


Fig. 5. Sensorgrams of ERp57, ERp57 pre-incubated with PDI (A), and ERp57 pre-incubated with CRT (B). The concentrations of pre-incubated analytes (shown in the inset) were injected over immobilized hCRT (A) or hPDI (B) on the sensor chip. The progress of analyte binding to immobilized hPDI and hCRT was monitored by following the increase in the signal [resonance units (RU)].

erone activity of PDI increased at low concentrations of added ERp57, while high concentrations suppressed the activity. Abundant levels of PDI localize to the lumen of ER [36]. Therefore, the quantity of ERp57 is lower than that of PDI in the ER, and the ratio of ERp57 to PDI should not be more than 1. These observations suggest that PDI and ERp57 affect the protein-folding process with other chaperone proteins.

The b and b′ domains of ERp57 are necessary for binding to the P domain of CRT [19–21]. The reported K_D value of ERp57 binding to CRT is approximately 10^{-6} M [37]. We obtained a similar K_D value of 3.15×10^{-6} M in this study (data not shown). Next, we examined whether PDI, ERp57, and CRT form a complex. Interestingly, when ERp57 bound to either PDI or CRT, it did not bind the other protein. Moreover, the binding manner of ERp57-PDI was distinct from that of ERp57-CRT, as observed with different sensorgrams (Fig. 5). These results collectively suggest that the complexes of PDI and ERp57 or CRT and ERp57 are competitively formed and sterically hinder each other.

In summary, we propose that ERp57 competitively binds to PDI and CRT to facilitate the folding of nascent proteins in the ER. In a previous report, PDI, Bip, and ERp72 were identified as part of a complex that aids in the folding of newly synthesized secretory

proteins [38,39]. However, this complex did not include ERp57. ERp57 binds to CRT in a 1:1 stoichiometry [37]. Currently, the number and identities of proteins required to assemble the entire complex with PDI and ERp57 remain to be determined. In this report, we demonstrate that ERp57 competitively forms complexes with chaperone proteins, such as PDI and CRT.

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