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PDI family protein ERp29 forms 1:1 complex with lectin chaperone calreticulin

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ABSTRACT

Lectin chaperone calreticulin is well known to interact with ERp57 which is one of PDI family proteins. The interaction of ERp57 with calreticulin is believed to assist disulfide bond formation of nascent glycoprotein in the ER. Various kinds of PDI family proteins are present in the ER, however, their precise roles have been unclear. In this study, interaction assay between PDI family proteins and calreticulin by SPR analysis was performed. Our analysis revealed for the first time formation of a 1:1 complex between ERp29 and calreticulin. The dissociation constant of interaction between ERp29 and calreticulin was shown to be almost identical to ERp57–calreticulin interaction. We speculate that the recognition site of ERp29 within calreticulin is different from that of ERp57.

1. Introduction

Various molecular chaperones in the endoplasmic reticulum (ER) have been known to assist protein folding of nascent *N*-glycoproteins. Especially, a protein quality control system by lectin chaperone calreticulin (CRT) and calnexin (CNX) minimizes formation and accumulation of misfolded proteins [1,2]. The ER luminal protein CRT consists of an N-domain possessing lectin active site and a P-domain composed of proline-rich repeat sequences [3]. In addition to its carbohydrate recognizing ability, the N-domain is able to interact with incompletely folded proteins [4], indicating that this domain plays an important role in glycoprotein folding [5]. It is generally accepted that P-domain interacts with thiol-disulfide oxidoreductase ERp57 [6], a structural homologue of protein disulfide isomerase (PDI) [7].

The nascent glycoprotein whose high-mannose type glycan is bound to glucose residue becomes substrate for CRT as shown in Fig. 1. In association with ERp57, CRT assists protein folding and formation of disulfide bonds in the substrate. The glucose residue of the glycan moiety of substrate dissociated from CRT is hydrolyzed by glucosidase II, and then the glycoprotein in properly folded state is produced. Zapun et al. reported that the redox activity of ERp57 measured by disulfide formation of ribonuclease B was enhanced depending on interaction with CNX/CRT [8]. Recently, interaction of CRT with cyclophilin B which is one of peptidyl prolyl *cis–trans*-isomerases has been found [9]. Furthermore, it is also indicated that this interaction, similarly to ERp57, is mediated via P-domain of CRT. These findings suggest that the lectin chaperone recruits several kinds of folding assistance proteins in order to cope with multifarious circumstances of glycoprotein maturation according to a situation of the substrate.

The PDI consists of 4 thioredoxin-like domains and an acidic carboxyl domain [10]. Although ERp57 also contains thioredoxin-like domains similarly to PDI, the acidic domain is replaced to cationic region [11]. There are characteristic CXXC motifs which include 2 cysteine residues in the thioredoxin-like domain, and the motif is indicated to have redox-active functions. Multiple kinds of PDI-resemble protein such as ERp27 and PDIp have been discovered in the ER [12]. Some of them were indeed indicated to be redox active similarly to PDI. In contrast, PDI-like proteins lacking CXXC motif are also known as shown in Table 1, while thioredoxin-like domain exists in the molecule. The biological relevance of diversity of PDI family protein *in vivo* has remained still unclear. In this study, investigation of protein–protein interaction between PDI family protein and CRT was carried out using SPR analysis. Our analysis found for the first time the interaction of CRT with ERp29 which contains no CXXC motif. It was also discovered that the affinity of CRT with ERp29 was almost same as that with ERp57.

Abbreviations: CNX, calnexin; CRT, calreticulin; PDI, protein disulfide isomerase; SPR, surface plasmon resonance.

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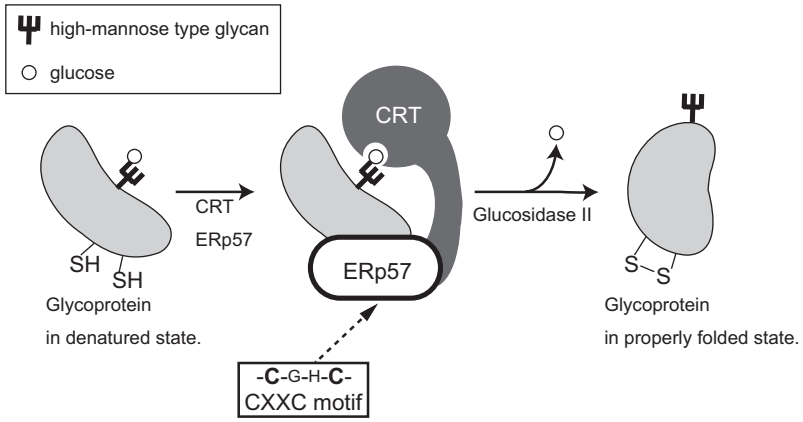

Fig. 1. Schematic illustration for glycoprotein folding assisted by ERp57–CRT complex.

Table 1
Estimated molecular weights of recombinant proteins and numbers of CXXC motif contained in single molecule of PDI family protein.

Recombinant protein	Molecular weight (kDa)	Number of CXXC motif
ERp27	29.4	0
ERp29	30.7	0
ERp44	46.7	1
ERp46	38.1	3
ERp57	58.0	2
PDI	58.0	2
PDIp	58.6	2
CRT	47.9	–

2. Materials and methods

2.1. Reagents

General reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). HEPES was obtained from Dojindo Co., Ltd. (Kumamoto, Japan). HBS-P buffer, CM5 sensor chip and amine coupling kit were purchased from GE healthcare (Piscataway, NJ).

2.2. Preparation of recombinant PDI family proteins and calreticulin

Recombinant proteins were produced by *Escherichia coli* expression system. A cDNA encoding 26–273 amino acids of ERp27 was cloned into pCold I expression plasmid (Takara Bio Inc., Otsu, Japan), which is designed to produce N-terminally $(His)_6$-tagged proteins. Similarly, an expression plasmid encoding 25–261 amino acids of ERp29, 29–406 amino acids of ERp44, 10–324 amino acids of ERp46, 25–505 amino acids of ERp57, 20–508 amino acids of PDI, 14–511 amino acids of PDIp or 24–417 amino acids of CRT was constructed. The obtained plasmid was transformed into BL21 cells, and the recombinant proteins were expressed and purified using Ni–NTA agarose (QIAGEN Gmbh, Hilden, Germany), according to the manufacturer's instructions. The obtained proteins were dialyzed in 10 mM HEPES buffer (pH 7.4) using Slide-A-Lyzer dialysis cassettes (10000 MWCO, PIERCE, IL, USA). The yields of ERp27, ERp29, ERp44, ERp46, ERp57, PDI, PDIp and CRT were 3.4, 4.3, 3.4, 2.7, 3.6, 2.5, 3.7, 1.3 mg from 1 L culture, respectively.

2.3. Affinity measurements using surface plasmon resonance

Affinity measurements by surface plasmon resonance (SPR) were performed in HBS-P buffer [10 mM HEPES (pH 7.4), 150 mM NaCl and 0.05% v/v Surfactant P20] including 1 mM $CaCl_2$ with a Biacore T100 system (GE Healthcare) at 25 °C. CRT was dissolved in 10 mM sodium acetate buffer (pH 4.5) to a final concentration of 0.1 mg/ml and immobilized on CM5 chip using standard amine coupling procedure. The response of CRT immobilization was approximately 1184 RU. A flow cell without calreticulin immobilization served as a control for nonspecific binding, and flow rate was maintained at 10 μl/min during immobilization. To conduct equilibrium binding measurement, various concentrations (5–150 μM) of PDI family proteins were injected into each flow cell at 10 μl/min for 2 min. Binding response was recorded as the difference between signal of control flow cell and one of the CRT-immobilized flow cells.

2.4. Binding kinetics analysis

Calculation of dissociation constant K_d values was performed assuming a 1:1 protein–protein interaction. Response levels at equilibrium were plotted against protein concentration and calculated via a non-linear fitting of the following binding equation (KaleidaGraph, Synergy Software, Inc.).

$$R_{eq} = R_{max}[\text{PDI family protein}]/(K_d + [\text{PDI family protein}])$$

R_{eq} and R_{max} indicate the response value in equilibrium and the maximum response, respectively.

3. Results and discussion

Fig. 2 shows SDS–PAGE profiles of purified PDI family proteins, whose molecular weights were in good agreement with ones estimated from their amino acid sequences (Table 1). As they were

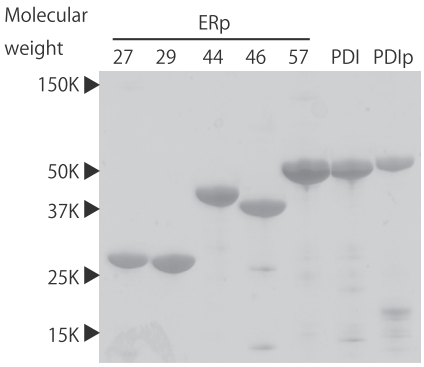

Fig. 2. SDS–PAGE profiles of recombinant PDI family proteins.

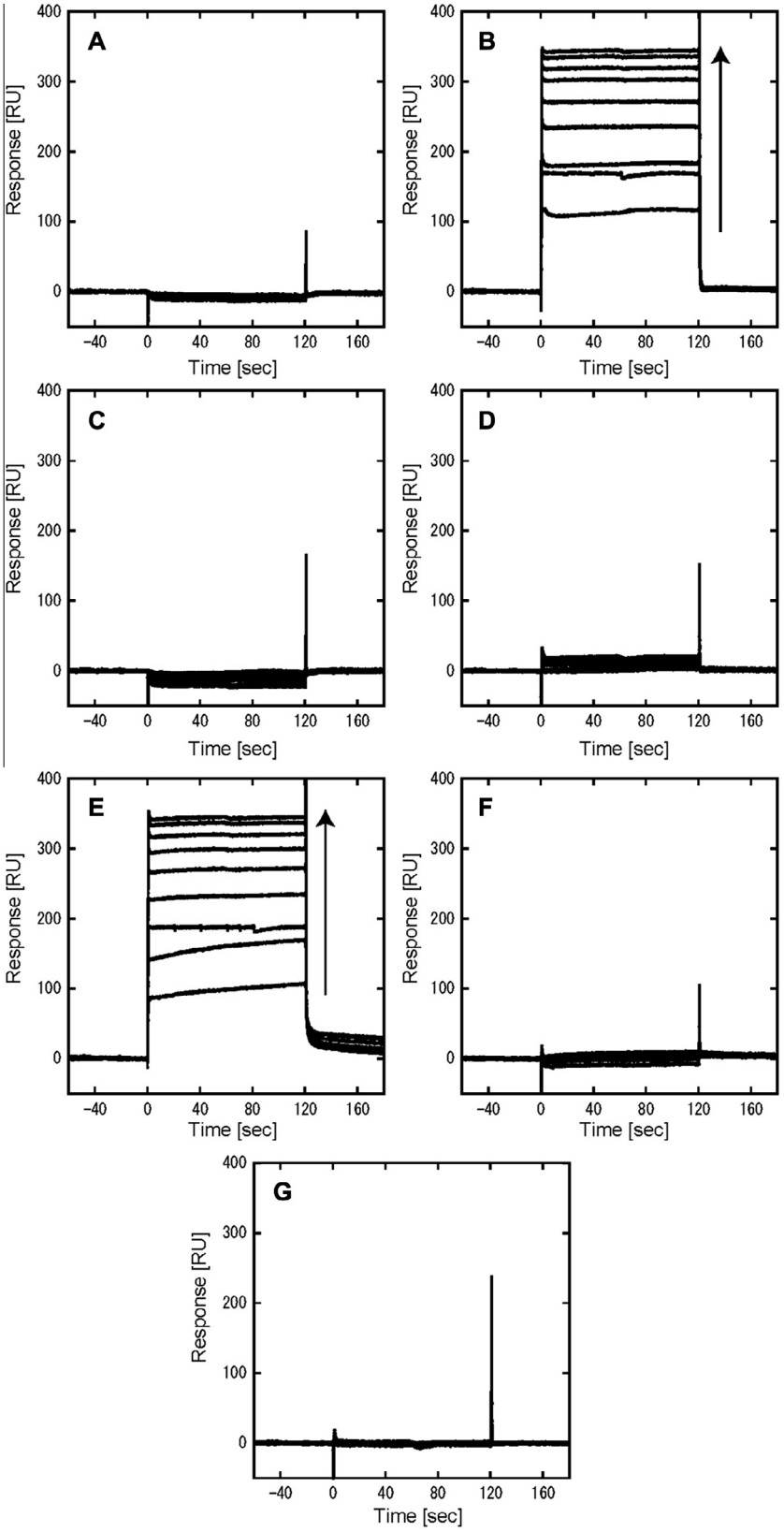

Fig. 3. SPR sensorgrams of PDI family protein binding to immobilized CRT on a CM5 sensor chip. Various concentrations (5–150 μM) of PDI proteins were injected for 120 s at a flow rate of 10 μl/min. The signal from a reference surface was subtracted from the response. PDI family proteins used in the SPR analysis were following; (A) ERp27, (B) ERp29, (C) ERp44, (D) ERp46, (E) ERp57, (F) PDI, and (G) PDIp.

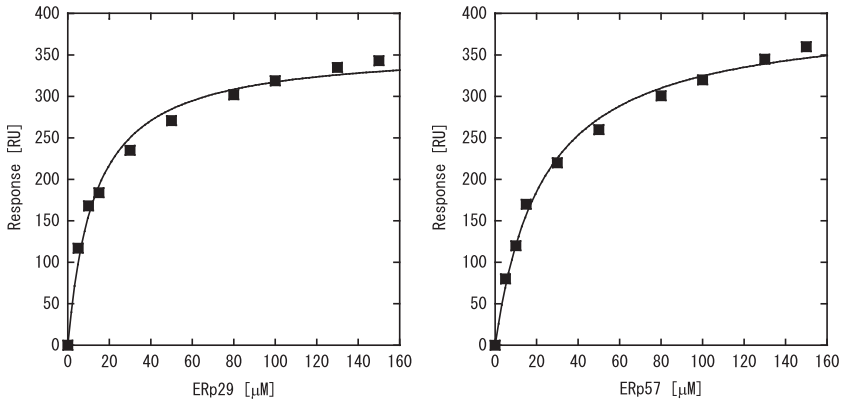

Fig. 4. Plots of response in equilibrium state against concentration of PDI protein. The continuous lines represent the fit to 1:1 binding equation.

detected as single bands, purified proteins suitable for protein–protein interaction assay were sufficiently obtained.

The interaction assays between CRT and PDI family proteins were performed using Biacore SPR system. Several concentrations of PDI proteins were injected into flow cell, and then interaction with CRT immobilized on sensor chip was measured. Fig. 3 indicates sensorgrams reflecting interaction of CRT with each PDI family protein. No appreciable change of response was observed in ERp27, ERp44, ERp46, PDI and PDIp. The results show that these proteins barely interact with CRT. In contrast, the sensorgrams relevant to ERp29 (Fig. 3(B)) and ERp57 (Fig. 3(E)) show increase of response depending on protein concentration, indicating that CRT possesses ability to interact with both proteins. The result of ERp57 is in good agreement to past findings [13]. The existence of interaction between PDI and CRT has been controversial in previous studies [13–17], however, our result indicates that PDI has little ability, if any, to interact with CRT.

Above interaction assay using SPR found interaction of CRT with ERp29 in addition to ERp57. Detailed information of CRT interaction with ERp29 and ERp57 were analyzed based on relationship between responses of SPR sensorgram and concentrations of each protein. The theoretical equation of 1:1 complex formation was fitted to the plots of response in equilibrium state against protein concentration (Fig. 4). These results indicated that the plots of ERp29 and ERp57 well matched with the line of theoretical equation. The complex formation between CRT and ERp57 by 1:1 binding has been known [18], which was corroborated by our result. Accordingly, 1:1 complex formation is strongly suggested in the interaction of ERp29 with CRT. Table 2 indicates the dissociation constants of each interaction estimated from the fitting curve. The K_d value of ERp29 and ERp57 were calculated to be 13.0 and 23.1 μM, respectively, indicating that the affinity of ERp29 for CRT is quite similar to ERp57. Frickel et al. reported that the K_d value between P-domain of ERp57 and CRT was about 18 ± 5 μM from TROSY-NMR analysis [18]. This K_d value is also in excellent agreement with our result, suggesting that the analysis of interaction by SPR was highly appropriate.

A total amount of each protein bound to CRT was investigated. The theoretical R_{max} means maximum response of ligand-analyte interaction when the recognition sites of all ligands immobilized on sensor chip are available to bind with analyte. However, R_{max} observed from experimental data has tendency to be lower than theoretical R_{max} since NHS/EDC method inevitably immobilizes proteins in a random manner. Table 2 indicates theoretical R_{max} of ERp29 and ERp57 estimated from the response of CRT during immobilization process. The theoretical R_{max} values of ERp29 and ERp57 were estimated to be 758.9 and 1433.7 RU, respectively. The difference of theoretical R_{max} value is presumably due to molecular weight of each PDI protein. Since the K_d value of each protein is almost same, the response of ERp57 is predicted to be higher than one of ERp29 when the recognition site of ERp57 in CRT is common to ERp29. However, the R_{max} of each protein estimated from fitting curve was almost identical (ERp29: 358.6 RU, ERp57: 399.8 RU). From the comparison of R_{max} value, 27.9% of total CRT immobilized on sensor chip was estimated to preserve ability to interact with ERp57. On the other hand, ERp29 was suggested to interact with the approximately half of immobilized CRT, indicating that the mode of interaction with CRT would be different between ERp57 and ERp29. This result suggests that they interact with CRT at different recognition site.

Table 2
Affinity constants of ERp29 and ERp57 with molecular chaperone calreticulin.

	K_d [μM]	Estimated theoretical R_{max}[a] [RU]	R_{max} calculated from SPR[b] [RU]	Ratio[c] [%]
ERp29	13.0	758.9	358.6	47.3
ERp57	23.1	1433.7	399.8	27.9

[a] R_{max} value estimated from response of CRT immobilization by following equation. Theoretical R_{max} = (MW of analyte/MW of CRT) × (response of CRT immobilization).
[b] R_{max} value calculated from curve fitting of binding equation.
[c] Ratio of calculated R_{max} value to theoretical R_{max} value.

In conclusion, we demonstrated for the first time the formation of 1:1 complex between ERp29 and CRT. It was indicated that the affinity of ERp29 to CRT was almost the same as that of ERp57. It is likely that the interaction manner of ERp29 with CRT differs from that of ERp57. Further study will clarify interaction mechanism of ERp29 with CRT and reveal the presence and the role of ERp29-CRT complex *in vivo*. Especially, since the CXXC motif is absent in ERp29 as shown in Table 1, its role as a binding partner of CRT is likely to be different from ERp57. Indeed, ERp29 has been reported to lack PDI-like folding assistance activity even if CRT is present [19]. It was also known that heterogeneous complex formation between ERp29 and thyroglobulin was observed in cell, suggesting the ERp29 involvement in a secretion process of thyroglobulin [20]. Therefore, the past findings suggest that ERp29 is associated with folding assistance in different manner from PDI. As a participation in tumorigenesis has been mentioned in a few reports [21,22], CRT-ERp29 complexation would become breakthrough to understand the enigmatic role of ERp29.

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