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Identification of the redox partners of ERdj5/JPDI, a PDI family member, from an animal tissue

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ABSTRACT

ERdj5 (also known as JPDI) is a member of PDI family conserved in higher eukaryotes. This protein possesses an N-terminal J domain and C-terminal four thioredoxin domains each having a redox active site motif. Despite the insights obtained at the cellular level on ERdj5, the role of this protein *in vivo* is still unclear. Here, we present a simple method to purify and identify the disulfide-linked complexes of this protein efficiently from a mouse tissue. By combining acid quenching and thiol-alkylation, we identified a number of potential redox partners of ERdj5 from the mouse epididymis. Further, we show that ERdj5 indeed interacted with two of the identified proteins via formation of intermolecular disulfide bond. Thus, this approach enabled us to detect and identify redox partners of a PDI family member from an animal tissue.

1. Introduction

Disulfide bonds are important structural feature of a great number of proteins that go through the secretory pathway. The enzymes that interact directly with folding proteins to introduce or break disulfide bonds are, mostly, oxidoreductases belonging to thioredoxin superfamily. The enzymes of this superfamily often have an active site containing a CXXC motif (cysteines separated by two amino acids) embedded in a thioredoxin-like fold [1].

In the endoplasmic reticulum (ER) of eukaryotes, proper formation of protein disulfide bonds is likely catalyzed directly by protein disulfide isomerase (PDI) family members. The PDI family members are characterized by the presence of one or more domains with thioredoxin-like fold, and their localization in the ER. There are ~20 PDI family members present in the ER of mammalian cells. Why so many PDI family members exist in the ER is unclear [2,3]. Insights into this question may be obtained by revealing the redox partners of each enzyme.

ERdj5 [also known as JPDI (J-domain containing PDI-like protein)] is a PDI family member that exists in the ER of mammalian cells. This protein possesses an N-terminal J-domain in addition to six C-terminal thioredoxin domains, four of which have CXXC active site motifs responsible for its disulfide exchange activity [4–8]. The J-domain of ERdj5 acts as a co-chaperone for the ER-resident Hsp70 (heat shock protein 70) chaperone, BiP (immunoglobulin heavy-chain-binding protein) [4–6].

Results obtained using model substrates suggest that ERdj5 plays an important role in the ER associated degradation (ERAD) of misfolded proteins [7,8]. During ERAD, misfolded proteins are first retrotranslocated from the luminal side of the ER to the cytosol, and are then degraded by the proteasome in the cytosol. Disulfide bridges formed in the substrate proteins may interfere with the movement of the substrate from the ER lumen to the cytosol through the unidentified retrotranslocon. Ushioda et al. [7] suggested that ERdj5 acts as a reductase that promotes the ERAD of misfolded proteins by breaking their disulfide bonds.

We have generated ERdj5 knockout mice to find that the absence of this enzyme causes induction of ER stress only in the salivary gland [9]. The weakness of the defect observed with the knockout mice implies that some other factor may substitute the function of this enzyme, which makes it difficult to know the physiological function of this protein at animal level.

Abbreviations: ER, endoplasmic reticulum; PDI, protein disulfide isomerase; ERAD, ER-associated degradation; NEM, N-ethylmaleimide; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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Recently, Oka et al. [10] identified a number of proteins that can form mixed-disulfides with ERdj5 using a variant of this enzyme expressed in a cultured cell line. With low-density lipoprotein receptor, one of the identified proteins, they showed that ERdj5 is required for the efficient folding of this protein in the cells, leading to a proposal that ERdj5 may also act as a reductase that promotes the oxidative protein folding. This finding together with other works [11–14] points to the usefulness of the information on the redox partners in understanding the roles of thioredoxin superfamily members.

Here, we identified the redox partners of ERdj5 from the mouse epididymis. To do this, we performed acid-quenching and modification of free cysteines of proteins from the tissue, followed by immuno-isolation using an antibody specific to ERdj5. Importantly, any genetic engineering was not required for this purpose. Accordingly, our results show that it is possible to identify the redox partners of a PDI family member from a tissue of a wild-type animal.

2. Materials and methods

2.1. Preparation of mouse tissue protein lysates alkylated with N-ethylmaleimide (NEM)

Eight-week old mice were used to prepare the tissues. A tissue, dissected from the body of a mouse, was disrupted in 1 ml of ice cold 10% trichloroacetic acid using a homogenizer (Polytron PT2020s) from Kinematica AG (Luzern, Switzerland). This step was completed within 10 s after the dissection. Following this step, we adopted a standard procedure for the alkylation of free cysteines [15]. Briefly, after 20 min incubation on ice, the proteins were collected by centrifugation, washed twice with ice cold acetone to remove acid, and dissolved in NEM alkylation buffer [100 mM Tris–HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 100 mM NEM] supplemented with 10 μg/ml pepstatin A, 10 μg /ml benzamidine, and 1 mM phenylmethylsulfonyl fluoride. To alkylate the proteins, the samples were agitated for 20 min and incubated for another 30 min at 37 °C. Mice were purchased from CLEA Japan and housed in specific pathogen-free facilities. All animal experiments were carried out in accordance with the policies of the Committee on Animal Research at Nara Institute of Science and Technology.

2.2. Antibodies

Guinea pig anti-ERdj5 antibody has been described [9]. Rabbit anti-ERp72 antibody was purchased from GeneTex (Irvine, CA). Mouse monoclonal anti-PDI antibody was obtained by the following procedure. Mice were immunized with the membrane fraction from unfertilized eggs of *Xenopus laevis* [16]. Hybridomas producing various antibodies were collected. We obtained one hybridoma, which secretes a specific monoclonal antibody that recognized both *Xenopus* and mammalian PDI. The ascites fluid from a mouse inoculated with the hybridoma was used as the source of the antibody.

The affinity beads used for the purification of ERdj5 were prepared using Protein A IgG Plus Orientation Kit (Pierce) and 1 mg of IgG purified from anti-ERdj5 antiserum.

2.3. Immunoblotting

The proteins from NEM-treated tissue lysates were separated by 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE), blotted onto an Immobilon-P membrane (Millipore), incubated with a primary antibody and then with an appropriate secondary antibody, and detected with Clarity Western ECL Substrate (Bio-Rad) and X-ray film or LAS4000 (Fuji Film).

2.4. Nonreducing, reducing two-dimensional gel electrophoresis

The NEM-alkylated protein lysate was first separated on a nonreducing 10% SDS–polyacrylamide gel prepared with spacers that have the thickness of 0.75 mm. The gel lane was cut out, incubated at 90 °C for 10 min in Laemmli SDS sample buffer containing 5% β-mercaptoethanol, and layered on top of the second dimension gel prepared using spacers with the thickness of 1 mm. After electrophoresis, the proteins were transferred onto an Immobilon-P membrane and detected as described above.

2.5. Affinity purification of ERdj5 and mixed-disulfide complexes that involve ERdj5

The NEM-alkylated lysate was denatured by incubation at 80 °C for 10 min, diluted tenfold with ice-cold KI buffer [2% Triton X-100, 50 mM Tris–HCl (pH 8), 150 mM NaCl, 1 mM EDTA], and centrifuged at 100,000×*g* for 10 min at 4 °C. The cleared lysate was incubated with anti-ERdj5 antibody conjugated with protein A agarose beads (see above) for 16 h at 4 °C. The immune complexes were collected by centrifugation, and washed six times with high salt buffer [1% Triton X-100, 50 mM Tris–HCl (pH 8), 1 M NaCl, 1 mM EDTA] and once with 10 mM Tris–HCl (pH 8). The immunoisolates were then released by incubating the sample at 90 °C for 3 min with Laemmli sample buffer.

2.6. Identification of redox partners of ERdj5 by mass spectrometry

One third of the total purified proteins obtained from thirteen mice were separated by 10% SDS–polyacrylamide gels, and the proteins that run slower than the position of the monomeric form of ERdj5 were excised from the gel, digested with trypsin and analyzed by liquid chromatography/tandem mass spectrometry (LCQ Advantage; Thermo Scientific) and peptide mass fingerprinting. When more than one unique peptide was read from a protein and the sequence coverage of the protein was more than 4.4%, we regarded the protein to be "identified". Affinity purification using conjugated antibodies often causes a portion of the conjugated antibodies to be released from the beads, resulting in their co-purification with the target proteins. We, thus, excluded immunoglobulins from the list of identified protein.

2.7. Immunoprecipitation

The NEM-treated epididymis lysate was incubated with the appropriate antibody or control serum for 2 h at 4 °C, and then with appropriate anti-Ig IP beads (eBioscience , San Diego, CA) for 1 h. Immuno-complexes formed were collected by centrifugation, and washed five times with IP buffer [50 mM Tris–HCl (pH 8), 150 mM NaCl, 1% NP-40] and once with 10 mM Tris–HCl (pH 8). The immunoisolates were then released by incubation with Laemmli sample buffer without reducing agent for 1 h on ice. The released proteins were incubated in the presence (reducing) or absence (nonreducing) of 50 mM dithiothreitol (DTT) for 10 min prior to SDS–PAGE. After SDS–PAGE, the gel was incubated in the presence of 200 mM β-mercaptoethanol for 30 min before protein transfer and subsequent immunoblotting (see above).

3. Results

3.1. Stabilizing disulfide-linked complexes formed in mouse tissues

Thiol-disulfide exchange reaction catalyzed by oxidoreductases including ERdj5 (Fig. 1A) likely proceeds through a disulfide-linked intermediate formed between the enzyme and its substrate

(Fig. 1B). Thus, the stabilization and purification of such complexes may lead to the identification of the redox partners. To stabilize disulfide-linked complexes, cells are often directly treated with 10% trichloroacetic acid to rapidly quench thiol-disulfide exchange reactions since protonation of thiolates of free cysteines inhibits the reactions [17]. For the efficient quenching of the exchange reactions and stabilization of the disulfide-linked enzyme-substrate complexes formed in an animal "tissue", we disrupted the tissue in 10% trichloroacetic acid using a homogenizer immediately after cutting off the tissue from a male mouse (Fig. 1C). Following this step, free cysteines in the acid-quenched sample were modified with an alkylation agent, N-ethylmaleimide (NEM), using a standard procedure, leading to the stabilization of the disulfide-linked complexes [15]. Use of this method, indeed, appears to have stabilized mixed-disulfide intermediates efficiently because a number of proteins identified as partners of ERdj5 by Oka et al. [10] using a variant of ERdj5 were also identified in this study (see below and Table 1).

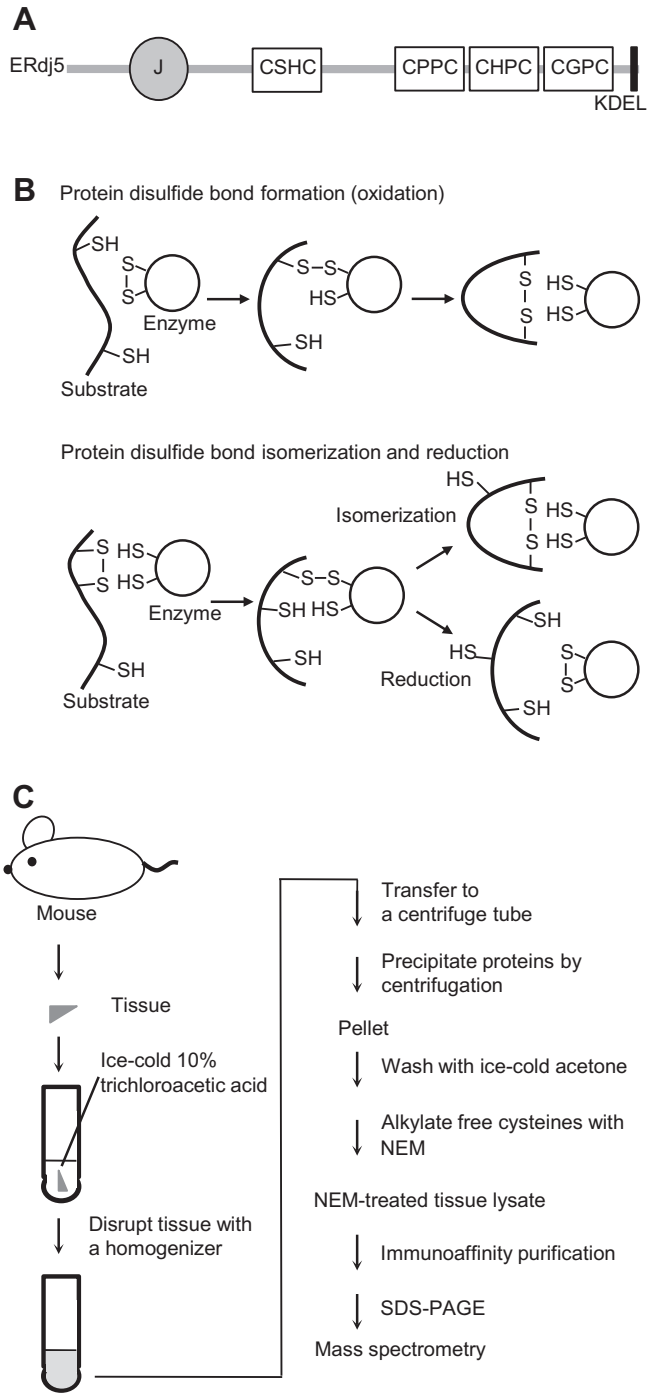

Fig. 1. Strategy for the identification of the redox partners of ERdj5. (A) Schematic representation of the domain organization of ERdj5. Open rectangles containing the active site sequence, thioredoxin domains with active sites; J, J-domain; KDEL, ER retrieval signal. (B) Mechanisms of thiol-disulfide exchange reactions catalyzed by thiol-disulfide oxidoreductases. Because thiol-disulfide exchange reaction is mediated by the attack on a disulfide by a thiolate anion, the thiol of the attacking cysteine must be deprotonated for the reaction to take place (not shown). Thus, strong acid inhibits the exchange reactions, resulting in the transient stabilization of the disulfide-linked complexes. (C) Purification of mixed-disulfide complexes that involve ERdj5 from a mouse tissue.

3.2. Disulfide-linked complexes that involve ERdj5 are abundant in epididymis and prostate

To detect ERdj5 and any disulfide-linked complexes that involve ERdj5, proteins from the NEM-treated lysates were separated by SDS–PAGE under non-reducing conditions and subjected to immunoblotting with anti-ERdj5 antibody. A band that corresponds to the monomeric form of ERdj5 was detected at ~85 kDa (Fig. 2A) [9]. Remarkably, with some tissues, we observed, in addition to this band, a number of bands of different molecular masses that reacted with antibody to ERdj5 (lanes 10–13). We suggest that latter bands represent disulfide-linked complexes formed between ERdj5 and its partners for two reasons. Firstly, they ran slower than the monomeric form of ERdj5. Secondly, when the samples were treated with reducing agent before electrophoresis, these bands disappeared giving rise to a band corresponding to the monomeric form of ERdj5 (Fig. 2B, lanes 10–13). Consistently, when the gel lane corresponding to the lane 10 of Fig. 2A was cut off from gel and run under "reducing" conditions, the higher bands also migrated at ~85 kDa in the second dimension (Fig. 3). Thus, we detected a number of disulfide-linked complexes that involve ERdj5 in NEM-treated samples prepared from mouse tissues.

We observed that the monomeric form of ERdj5 and complexes that involve ERdj5 were abundant in genital organs including the epididymis, prostate, testis, and seminal vesicle. Consistently, it has been reported that ERdj5 mRNAs are highly expressed in the prostate, testis, and epididymis [4]. Although both male and female ERdj5 knockout mice were fertile and could breed to produce progeny [9], these findings imply that ERdj5 plays some specific role in these tissues.

Table 1
Identified proteins.[a]

Names	Accession No.	Known partner	Cys[b]	Coverage[c] (%)	Location
ERp57 (PDIA3)	NP_031978	Yes	7	49.1	ER
P5 (PDIA6)	NP_082235	Yes	6	38.4	ER
ADAM7	NP_031428		44	38.1	PM[d]
PDI (PDIA1)	P09103	Yes	6	27.5	ER
ERp72 (PDIA4)	P08003	Yes	6	27.3	ER
Gpx5	NP_034473		4	23.1	Secreted
ERp44	NP_083848	Yes	6	16.5	ERGIC[e]
Ovch2	NP_766496		19	12.6	Secreted
Adam28	NP_034212		44	12.1	PM
Mia3	NP_796363		11	6.7	ER exit sites
Grp94	AAH10445	Yes	3	4.6	ER
Ces5a	NP_001003951		8	4.5	Secreted

[a] ERdj5 itself is omitted from this list.
[b] The number of cysteine residue in the mature polypeptide.
[c] The value of percent coverage.
[d] PM, plasma membrane.
[e] ERGIC, ER-to-Golgi intermediate compartment.

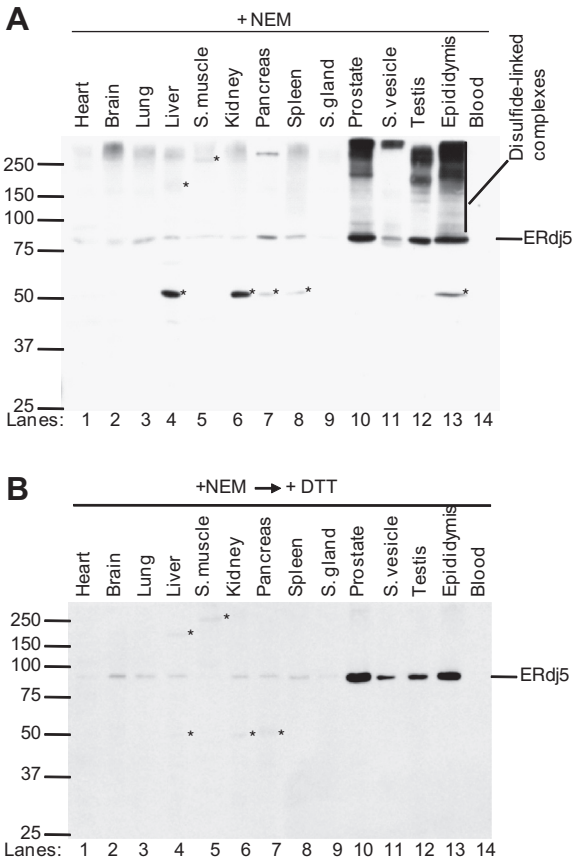

Fig. 2. Tissue distribution of ERdj5 and the mixed-disulfide complexes that involve ERdj5. (A and B) Proteins from NEM-treated mouse tissue lysates were separated by SDS–PAGE, and visualized by immunoblotting with antibody to ERdj5. Each lane contains 5 μg of proteins from the indicated tissue. In panel B, the samples were incubated with 50 mM dithiothreitol before electrophoresis. The positions of marker proteins are indicated on the left in kDa. The positions of the monomeric form of ERdj5 are indicated on the right. Non-specific bands are marked with asterisks. S. muscle, skeletal muscle; S. gland, submandibular gland; S. vesicle, seminal vesicle.

3.3. Purification and identification of the redox partners of ERdj5 from mouse epididymis

To identify the redox partners of ERdj5, we purified the ERdj5-containing complexes from the epididymis because ERdj5 was abundant in the tissue. To do this, the NEM-treated epididymis lysate was incubated with affinity-beads conjugated with anti-ERdj5 antibody. After washing the immuno-complexes intensively, we released the immuno-isolated materials from the beads by incubating the beads in Laemmli sample buffer. The eluted proteins were then separated by SDS–PAGE. Although one third of the total eluted proteins processed from thirteen mice were loaded into the well of a gel, we failed to detect the immuno-isolated proteins with coomassie brilliant blue staining (not shown). Since silver staining can interfere with peptide mass spectrometry [18], and the complexes were detected as smeared bands (Fig. 2A, lane 13), we directly cut off, without silver staining, the area of the gel lane corresponding to the disulfide-linked complexes, digested the proteins in the gel with trypsin and identified the released peptides by mass spectrometry (Table 1).

The identified proteins were those that reside in the ER or those on the secretory pathway (Table 1). In addition, each of them has more than one cysteine (Table 1), consistent with that they represent the products of some disulfide-mediated interactions that occurred between ERdj5 and its redox partners in the ER.

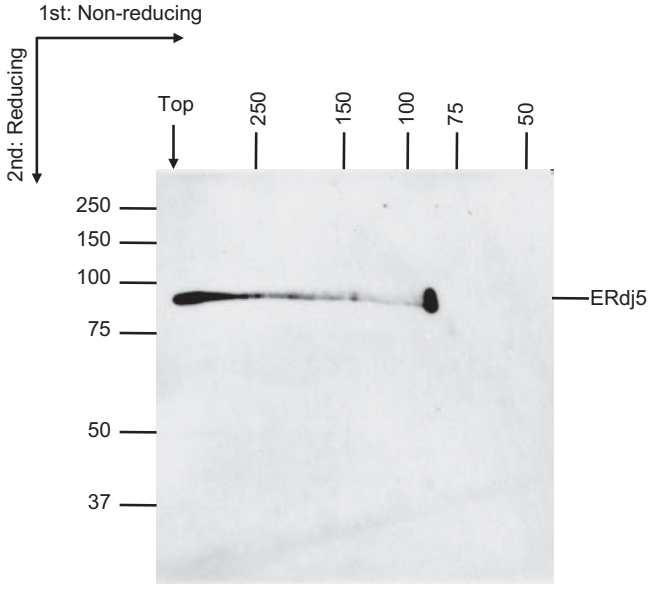

Fig. 3. ERdj5 forms disulfide-linked complexes having a variety of apparent molecular masses in a mouse tissue. Proteins from a NEM-treated mouse prostate lysate were separated by non-reducing-reducing two-dimensional SDS–PAGE and subjected to immunoblotting with antibody to ERdj5. The gel was run for longer time for better separation in the first dimension. The positions of marker proteins are indicated on the top and left in kDa. The position of monomeric form of ERdj5 is indicated on the right.

3.4. ERdj5 interacts with other PDI family members via intermolecular disulfide bond in epididymis

To characterize the interaction between ERdj5 and the identified proteins, we immunoprecipitated ERp72 [2] from the lysate using antibody to ERp72. The immunoprecipitates were then subjected to immunoblotting using anti-ERdj5 antibody. Separation of the immunoprecipitates using non-reducing gel revealed the presence of a faint but distinct band at ~160 kDa (Fig. 4A, lane 3). We suggest that this band represents a disulfide-linked complex formed between ERdj5 and ERp72 for the following reasons. Firstly, this band was immunoprecipitated with antibody against ERp72. Secondly, this band is specifically recognized by antibody against anti-ERdj5 antibody (Fig. 4A, lane 3). Thirdly, the apparent molecular mass of the band (~160 kDa) agrees well with the sum of the molecular masses of ERdj5 (87 kDa) and ERp72 (70 kDa) (Fig. 4A, lane 3). Finally, when the sample was treated with reducing agent before electrophoresis, this band disappeared giving rise to a band representing the monomeric form of ERdj5 (Fig. 4A, lane 6). These findings support the view that the interaction between ERdj5 and ERp72 is mediated by intermolecular disulfide bond.

In a similar experiment, use of antibody to PDI for immunoprecipitation allowed us to detect total of three mixed-disulfide complexes formed between ERdj5 and PDI (Fig. 4B, lane 3). The smallest of the complexes likely represents a heterodimer of ERdj5 and PDI because its apparent molecular mass (~150 kDa) agrees well with the sum of the molecular masses of ERdj5 (87 kDa) and PDI (55 kDa). The other two bands may represent mixed-disulfide complexes that contain more than two proteins as they run slower than the presumed heterodimer. Thus, this strategy allowed us to identify redox partners of ERdj5 from an animal tissue.

4. Discussion

Mammalian ER possesses ~20 PDI family members, some of which are presumed to perform disulfide exchange reactions with

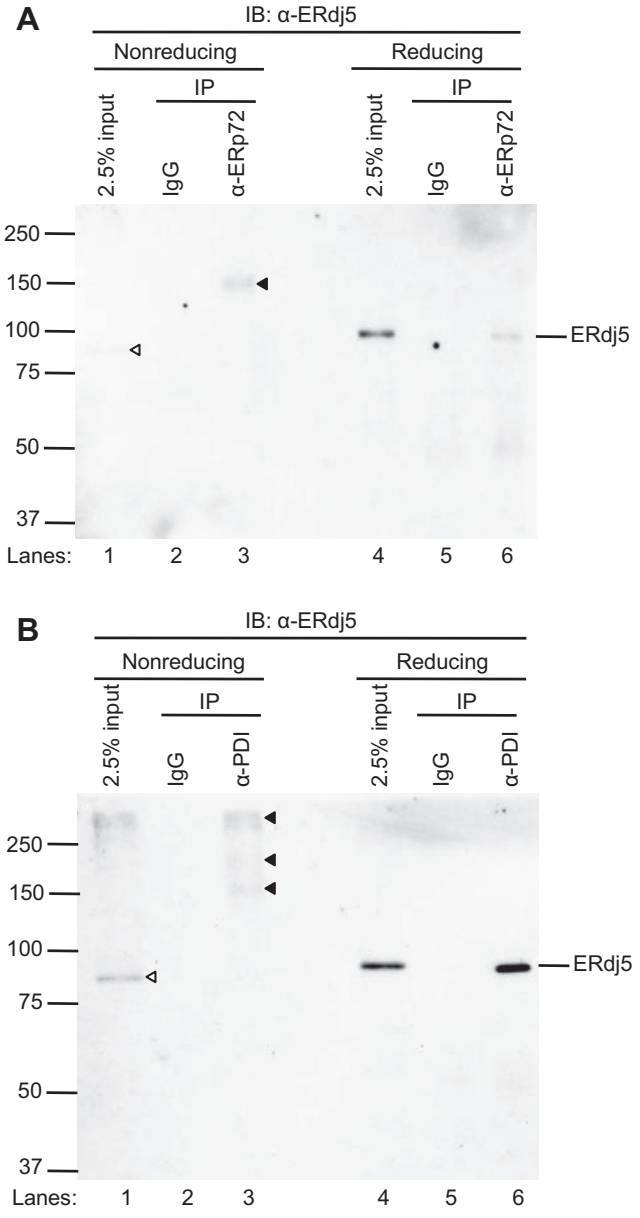

Fig. 4. Detection of disulfide-linked complexes formed between ERdj5 and two of the identified partners of this protein in NEM-treated epididymis lysate. (A) Immunoprecipitates from NEM-treated epididymis lysate obtained using a rabbit antibody to ERp72 (lanes 3 and 6) or using a control rabbit serum (lanes 2 and 5) were subjected to immunoblotting with an antibody to ERdj5 to detect the complex formed between ERp72 and ERdj5. Lanes 1 and 4 contain 1 μg of NEM-treated epididymis lysate. Lanes 2, 3, 5, and 6 contain immunoprecipitates obtained from 40 μg of NEM-treated epididymis lysate. The samples were reduced with 50 mM DTT before electrophoresis in lanes 4–6. The closed arrowhead depicts a disulfide-linked complex formed between ERdj5 and ERp72. The position of the monomeric form of ERdj5 is indicated on the right. (B) The experiments were performed in the same manner as described in panel A except that NEM-treated epididymis lysate was first subjected to immunoprecipitation using an antibody to PDI (lanes 3 and 6). In lanes 2 and 5, a mouse control serum was used as an antibody for immunoprecipitation. The closed arrowheads indicate the positions of the disulfide-linked complexes formed between ERdj5 and PDI. (A and B) Note that the monomeric form of ERdj5 runs slightly faster under non-reducing conditions (lane 1) (open arrowheads) than does under reducing conditions.

ER proteins or proteins undergoing biosynthesis in this cellular compartment. Despite the potential importance of these enzymes for animals, their role is not well studied at the tissue or animal level. During disulfide exchange reactions, PDI family members form mixed-disulfide complexes with their target proteins. Here, we have combined acid-quenching and free cysteine modification to stabilize the disulfide-linked complexes that involve ERdj5 and identified its redox partners from an animal tissue.

In this study, we have identified a number of PDI family members (ERp57, P5, PDI, ERp72, and ERp44) as presumed redox partners of ERdj5 in the epididymis (Table 1). At least two of them (ERp72, and PDI) interacted with ERdj5 via intermolecular disulfide bond. Thus, these two proteins are indeed redox partners of ERdj5. Recently, Oka et al. [10] also identified these PDI family members as potential redox partners of ERdj5 in their experiments using a variant of this enzyme expressed in a cultured cell line. Thus, these PDI family members appear to be common redox partners of ERdj5.

Then, why do so many different PDI family members interact with ERdj5? One of the possibilities is that, by forming the mixed-disulfide complexes, ERdj5 and other PDI family members may exchange disulfide bond, thereby regulating their activities. Since *in vitro* studies suggest that ERdj5 is unique in that this enzyme likely acts as a reductase of disulfide bonds [5,7], this protein might be well suited to regulate the activities of other enzymes. Apparently, further study is needed to clarify the role of these interactions.

It has been assumed that reduction or isomerization of disulfide bonds in a target protein by thiol-disulfide oxidoreductases including PDI starts by their attacking on the disulfide bond on the target protein, leading to the formation of a mixed-disulfide complex formed between the enzyme and its substrate protein (see "Isomerization" and "Reduction" in Fig. 1B). This can be followed by the attack of the second cysteine of the active site of the enzyme on the intermolecular disulfide bond, releasing the enzyme and the substrate. Mutating the second cysteine of the active site of the enzyme to alanine or serine blocks the latter reaction, leading to the stabilization of the disulfide-linked complex between the enzyme and the substrate. Using this property of the enzymes, researchers have expressed the active site mutant of enzyme to stabilize the complex, leading to the detection and identification of the targets of some thioredoxin superfamily members [11–12,14]. In fact, as described above, Oka et al. [10] identified the redox partners of ERdj5 from a cultured cell line using exogenously expressed variant of ERdj5 lacking the C-terminal cysteine of the active site on the assumption that ERdj5 acts as a reductase of disulfide bonds. Thus, the method is effective in identification of the redox partners of reductases or isomerases.

However, there are at least two limitations with the above strategy. Firstly, we need to express the variant of the enzyme exogenously to use the method. Secondly, the method is applicable to reductase or isomerase but not to oxidase since the active site of oxidase need to be disulfide bonded to interact with its substrate and initiate the oxidation reaction (Fig. 1B).

Here, we showed that, at least with ERdj5, it is possible to detect and identify the disulfide-linked complexes formed between this enzyme and its redox partners in a mouse tissue. By simply disrupting a tissue in acid and then modifying free cysteines with NEM using a standard protocol, we were able to detect the disulfide-linked complexes that involve ERdj5 and identify its redox partners. Importantly, we detected the disulfide-linked complexes formed by the endogenous enzyme and did not need to use a variant of this enzyme. Thus, this approach may allow the application of proteomics technology to identify the redox partners of ERdj5 in animal tissues, and, perhaps, those of the other PDI family members, and thereby may help define the role of the enzyme(s) at animal or tissues level.

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