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Oligomerization properties of ERp29, an endoplasmic reticulum stress protein

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Abstract ERp29, a novel and ubiquitously expressed endoplasmic reticulum (ER) stress-inducible protein, was recently isolated and cDNA cloned in our laboratory. Using size exclusion chromatography and chemical cross-linking we have assessed the oligomerization properties of ERp29. Purified ERp29 in solution as well as in rat hepatoma cells self-associates predominantly into homodimers. Labeling of the cells with [^{35}S]methionine with subsequent cross-linking and immunoprecipitation showed that ERp29 interacts with a number of ER proteins, one of which was previously identified as BiP/GRP78. Secondary structure prediction and fold recognition methods indicate that the native conformation of ERp29 resembles the thioredoxin fold, a structural motif characteristic of a number of enzymes with the redox function, including protein disulfide isomerase (with which ERp29 shares limited sequence similarity). Dimerization of the protein is suggested to be advantageous for the protein binding potential of ERp29.

Key words: ERp29; Protein disulfide isomerase; Molecular chaperone; Cross-linking; Size exclusion chromatography; Oligomer

1. Introduction

Cells respond to a variety of environmental stresses including physical (e.g. heat) or chemical (e.g. heavy metals, organic solvents) impacts by increased transcription of genes encoding so called heat shock or stress proteins. It is widely assumed that the common cell damaging mechanism for cellular stress factors is an overaccumulation of malfolded, denatured or aggregated proteins in the cytoplasm and luminal compartment of the endoplasmic reticulum (ER) [1,2]. The protective action of stress proteins under these conditions is accomplished by binding to denatured or aggregated cellular proteins thereby facilitating their refolding. Many stress proteins are constitutively expressed and function as molecular chaperones, i.e. assist in folding and block aggregation of nascent polypeptide chains by preventing incorrect intra- and intermolecular interactions. Other folding enzymes, such as protein disulfide isomerase (PDI) and peptidylprolyl isomerase, are also involved in maturation of proteins. PDI is of particular importance for protein folding in the ER where it catalyzes the formation and isomerization of disulfide bonds [3,4] and it has also been proposed to function as a molecular chaperone [5–7].

Recently a novel ER stress-inducible protein, ERp29, with a limited similarity to the eukaryotic protein disulfide isomerases was isolated from the rat liver and cDNA cloned in our laboratory [8,9] and by others [10]. The ERp29 gene was found to be ubiquitously expressed and was activated in response to ER stress, i.e. accumulation of transport-incompetent, misfolded and/or underglycosylated secretory proteins. The ERp29 precursor is processed by a signal peptidase producing a mature polypeptide with a predicted molecular mass of 24.5 kDa, which is targeted to the luminal compartment of ER.

Our preliminary results [8] indicate that ERp29 co-immunoprecipitates with BiP/GRP78, an abundant ER-resident molecular chaperone, and moreover this association is enhanced by tunicamycin, a potent agent causing ER stress. Typically many luminal stress proteins/chaperones are found in vivo in such transient heteropolymeric complexes with misfolded proteins and with each other. Some of them may also form stable homooligomers [11–13].

In the present report we utilized size exclusion chromatography and chemical cross-linking to study oligomerization and protein binding properties of ERp29 in vitro and in intact cells.

2. Materials and methods

2.1. Protein expression and purification

His_6-tagged ERp29 cDNA was expressed in *Escherichia coli* as described earlier [8]. Briefly, a coding region of ERp29 cDNA (nucleotides 44–826) excluding the leader sequence was subcloned into the pQE30 expression vector (Qiagen) that adds six histidines to the N-terminus of the protein. Recombinant ERp29 was expressed in *E. coli* strain JM109 and large amounts of the protein were purified under non-denaturing conditions on Ni-NTA-agarose affinity column according to the manufacturer's recommendations (Qiagen).

2.2. Size exclusion chromatography

For size exclusion chromatography, Ultrogel AC44 (Pharmacia-LKB) was equilibrated in $NaCl/P_i$ (8 mM NaH_2PO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, 300 mM NaCl, pH 7.4). The column was calibrated by bovine serum albumin (68 kDa), ovalbumin (47 kDa) and chemotrypsinogen (27 kDa). The void volume was monitored in each run by dextran blue (2000 kDa). Recombinant ERp29 (0.5 mg) was applied in a volume of 500 μl with indicated supplements. The protein was eluted by $NaCl/P_i$ with the same additives at a flow rate of 8 ml/h and 1.4 ml fractions were collected. The elution was monitored by absorbance at 280 nm.

2.3. Electrophoresis and Western blot

Denaturing SDS-PAGE was carried out according to Laemmli [14]. Protein bands were detected with Coomassie brilliant blue R-250.

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Abbreviations: ER, endoplasmic reticulum; PDI, protein disulfide isomerase; PMSF, phenylmethylsulfonyl fluoride

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For immunoblot analysis, the electrophoretically separated proteins were transferred to a nitrocellulose membrane and probed with polyclonal antibodies raised against recombinant ERp29 as described earlier [8]. Immunoreactive bands were visualized by enhanced chemiluminescence according to the manufacturer's specifications (Amersham).

2.4. Chemical cross-linking

For cross-linking in solution, ERp29 was incubated in 40 mM HEPES, 0.2 M NaCl, pH 7.4 with various concentrations of a water-soluble, uncleavable, homobifunctional cross-linker, BS^3 (Pierce), at room temperature for 10 min. The reaction was stopped by adding 50 mM Tris-HCl, pH 8.0 and the samples were incubated on ice for 10 min before processing by SDS-PAGE and Coomassie blue staining. Alternatively ERp29 was cross-linked in the same buffer with glutaraldehyde or dithiobis(succinimidyl propionate) (DSP; Pierce).

FAO cells (a rat hepatoma line obtained from M. Weiss, Pasteur Institute, Paris, France) were grown, cross-linked with DSP on ice for 30 min and lysed as described elsewhere [8]. Cell lysates were processed by SDS-PAGE with subsequent immunoblotting. In another set of experiments, the cells were labeled for 3 h with [^{35}S]methionine (Amersham), cross-linked with DSP and lysed. ERp29 was immunoprecipitated from the cell lysates and immune complexes were analyzed by SDS-PAGE with subsequent autoradiography.

2.5. Structure predictions

Computer-assisted secondary structure and fold recognition predictions were accomplished using a system of neural networks, PHDsec [15], and fold recognition method 'gonnet+predd' [16] available at http://www.doe-mbi.ucla.edu/people/frsvr/frsvr.html.

3. Results

3.1. Analysis of purified ERp29 by size exclusion chromatography

The mature form of ERp29 was overexpressed in *E. coli* as a His-tagged fusion protein and purified to apparent homogeneity as described previously [8]. Size exclusion chromatography was used to determine the native molecular mass of the recombinant protein. The protein was eluted as a sharp peak in the fractions corresponding to a molecular mass of ~55 kDa, whereas no absorbance at 280 nm was registered in the fractions corresponding to the molecular mass of the monomeric form of mature ERp29 (24.5 kDa) (Fig. 1A). This suggests that at the concentration of about 1 mg/ml, ERp29 in solution exists predominantly as a dimer. Some additional small peaks eluting earlier than the dimer were also detected, suggesting that a minor portion of the ERp29 molecules are assembled in higher-order multimers. In order to assess the nature of forces stabilizing the oligomeric forms of ERp29, the protein was incubated with a non-ionic detergent (0.5% NP-40) or 1 M NaCl and subjected to chromatography. As evident from the elution profile (Fig. 1B,C) none of these compounds was able to shift the equilibrium towards the formation of monomeric species or cause dissociation of the oligomers. Divalent cations such as Ca^{2+} and Mg^{2+} were also ineffective in this sense (results not shown).

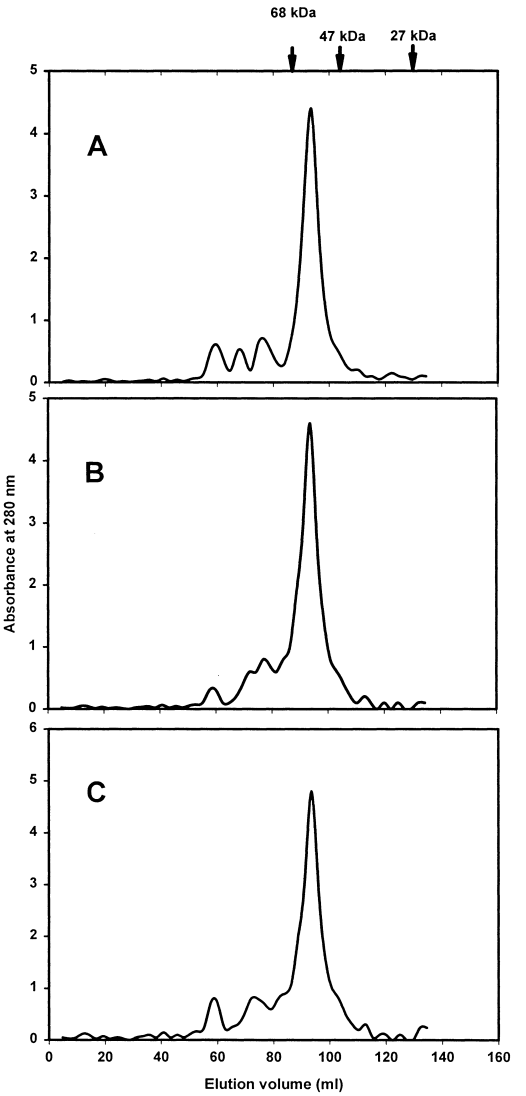

Fig. 1. Size exclusion chromatography of purified ERp29. Recombinant ERp29 (1 mg/ml) was applied on Ultrogel AC44 column and eluted as described in Section 2. Presented are fractions after the void volume. The column was calibrated using molecular mass standards bovine serum albumin (68 kDa), ovalbumin (47 kDa) and chemotrypsinogen (27 kDa). A: No additions. B: 1 M NaCl. C: 0.5% NP-40.

3.2. Analysis of multiple species of purified ERp29 by cross-linking

To obtain further information on the nature and proportion of ERp29 species in solution, the protein samples were subjected to cross-linking utilizing the non-cleavable homobifunctional cross-linker BS^3. Cross-linked species were then resolved by denaturing PAGE and stained with Coomassie blue. The results presented in Fig. 2A are essentially consistent with the chromatography data. ERp29 molecules at a concentration of 0.2 mg/ml are cross-linked in a dimer with a minor fraction assembled in multimers. Interestingly, even in the non-cross-linked sample (Fig. 2A, lane 1) we observed a faint band corresponding to the molecular mass of the dimer, indicating that even under denaturing conditions of SDS-PAGE, a small amount of the dimer molecules remains intact. Immunoblotting of the same samples demonstrated immunostaining of this 50 kDa band, verifying its identity as a dimer of ERp29 (results not shown). There are few reports in the literature where dimeric proteins have been found resistant to dissociation under the conditions of SDS-PAGE [17,18], apparently due to the very high binding constants of dimeric interactions [19]. The relatively broad banding pattern of cross-linked samples and the appearance of the second lower band apparently attribute to some heterogeneity in the intra-

molecular cross-linking. Under the same conditions the molecular mass of ovalbumin remains unchanged, indicating the specificity of cross-linking (Fig. 2B).

The dimeric form of ERp29 could be chased by increasing concentrations of BS^3 to the higher-order oligomers and some of them were even too large to be resolved by a 12% SDS gel (Fig. 2A, lanes 2–6). Apparently high concentrations of BS^3 caused non-specific extensive cross-linking of dimeric species of ERp29. A similar trend was seen in the samples with high, non-physiological concentration of ERp29 (Fig. 2C, lane 3). SDS-PAGE analysis under non-reducing conditions demonstrated an identical migration of the ERp29 band (Fig. 2D), which is a clear indication of a non-disulfide nature of bonds linking ERp29 molecules in the dimer. The additional ~60 kDa band (Fig. 2D, lane 2) may represent the native oligomeric form of a minor bacterial contaminant with disulfide bridge(s) (e.g. ~28 kDa polypeptide is visible in lane 1 but absent under non-reducing conditions (lane 2)). As in the case of size exclusion chromatography, neither high salt nor the detergent could dissociate the oligomers of ERp29 (Fig. 2A, lanes 7 and 8). These results were also confirmed using other cross-linkers, such as glutaraldehyde and DSP (data not shown).

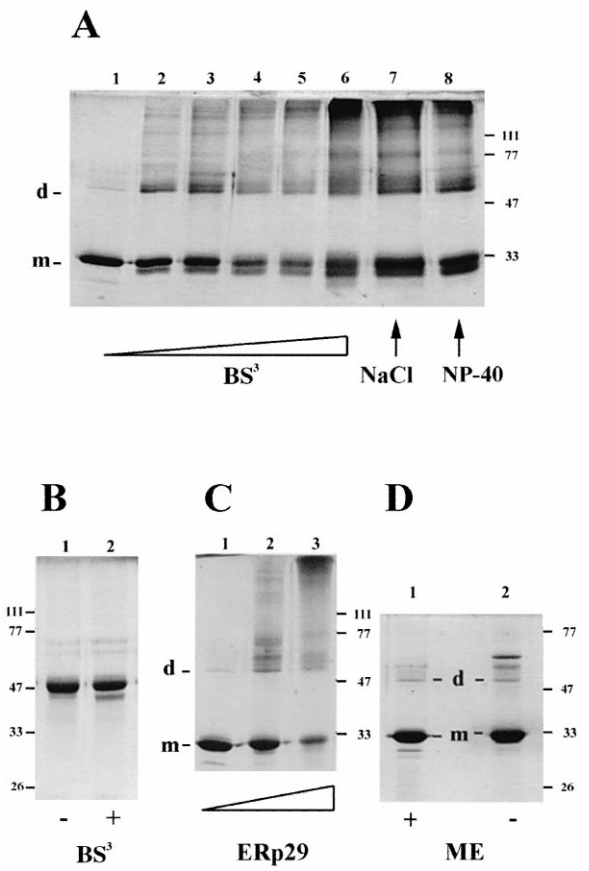

Fig. 2. Cross-linking of purified ERp29. A: Recombinant ERp29 (0.2 mg/ml) was cross-linked by BS^3 and samples were processed by SDS-PAGE with gels stained with Coomassie blue. Lane 1, control; lanes 20-6, 0.05–10 mM BS^3; ERp29 was cross-linked by 0.1 mM BS^3 in the presence of 1 M NaCl (lane 7) and 0.5% NP-40 (lane 8). B: Ovalbumin (1 mg/ml) was cross-linked under identical conditions by 0.1 mM BS^3 (lane 2). C: Cross-linking of various concentrations of ERp29 by 0.05 mM BS^3 (lane 1, 0.2 mg/ml; lane 2, 2 mg/ml; lane 3, 10 mg/ml). The same amount of protein (3 μg) was applied to each well. D: SDS-PAGE of ERp29 (0.2 mg/ml) under reducing (lane 1) and non-reducing conditions (lane 2). m, monomer; d, dimer.

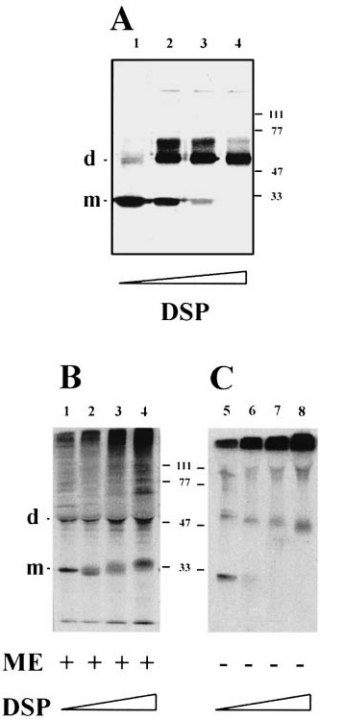

Fig. 3. Cross-linking in FAO cells. A: Cells were treated with: vehicle (Me_2SO) (lane 1) or 0.1–1 mM DSP (lanes 2–4). After cross-linking cells were lysed and processed by non-reducing SDS-PAGE and immunoblotting using polyclonal ERp29 antibodies. B: Cells were labeled with [^{35}S]methionine and treated with Me_2SO (lane 1) or 0.1–1 mM DSP (lanes 2–4) before lysis. Lysates from equal numbers of cells were immunoprecipitated with ERp29 antibodies and immune complexes resolved by non-reducing SDS-PAGE with subsequent autoradiography. C: Same as in B, but samples were run under reducing conditions. m, monomer; d, dimer.

3.3. Chemical cross-linking in the FAO hepatoma cells

In order to examine the oligomerization of ERp29 in intact cells, FAO hepatoma cells were cross-linked by a cell-permeable, thiol-cleavable agent, DSP, lysed and immunoblotted with anti-ERp29 serum. Under non-reducing conditions of SDS-PAGE, which maintains complexes linked by DSP intact, the amount of ERp29 oligomers (mostly in the form of a dimer) was increasing in a dose-dependent manner (Fig. 3A). The appearance of the band migrating closely to the dimer (Fig. 3A, lanes 2–4) may be interpreted as a result of the intramolecular cross-linking. No oligomeric complexes were detected when samples were run under reducing conditions (data not shown). Immunoprecipitation of ERp29 from the ^{35}S-labeled cells revealed a number of co-immunoprecipitating polypeptides including an appreciable amount of the non-denatured dimer (Fig. 3B, lane 1). Cross-linking selectively enhances the amount of some co-immunoprecipitated proteins (e.g. ~111 kDa and higher, 78 kDa (previously identified as BiP/GRP78, an abundant ER chaperone [8]), 65 kDa and 50 kDa migrating closely to the dimer) which appear to be the most probable interacting partners of ERp29 (Fig. 3B, lanes 2–4). However, the dose-dependent accumulation of the high molecular species as well as slight increase in the intensity of the dimer band may also reflect incomplete cleavage of

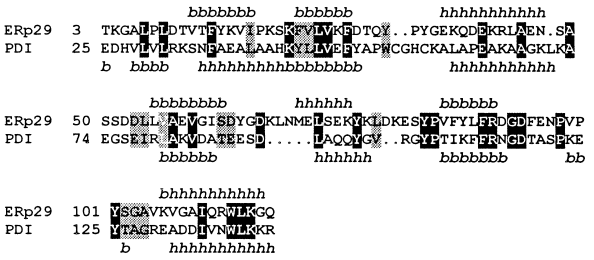

Fig. 4. Alignment of the N-domain of ERp29 with domain a of PDI and secondary structure prediction. The output from the fold recognition program was modified to highlight identical amino acids (dark shading) and conserved changes (lighter shading). Numbering of the ERp29 sequence starts from the beginning of the mature polypeptide. PDI sequence numbering starts from the precursor's first amino acid. b, β-sheet; h, α-helix.

the DSP-formed complexes by mercaptoethanol. Under non-reducing conditions these complexes remain uncleaved which results in the amassing of large oligomers (Fig. 3C, lanes 6–8). ERp29 appears to be involved in some of them as judged by the detection of the ERp29-immunoreactive high molecular mass bands under non-reducing conditions in the cross-linked samples of FAO cells (Fig. 3A, lanes 2–4). The appearance of some high molecular mass material even in the non-cross-linked sample (Fig. 3C, lane 5) under non-reducing conditions is apparently due to the presence of co-immunoprecipitating polypeptides containing S-S bonds that are cleaved under reducing conditions.

Thus, cell culture experiments confirm that ERp29 exists mainly as a dimer in intact cells and may also be involved in some high-order homo- and/or heterocomplexes.

4. Discussion

Our data suggest that at micromolar concentrations in solution, as well as in intact cells, ERp29 has a well-defined quaternary structure with two ERp29 molecules assembled in a homodimer. However, as was shown by the cross-linking of the recombinant protein, ERp29 at high concentrations may also form very large aggregates. Considering the abundant accumulation of ERp29 under the stress conditions [8], we hypothesize that this feature of ERp29 might be essential for the putative stress defense-related function of ERp29. A similar functional significance of oligomerization was observed, for instance, in the binding of cohort protein GrpE to the bacterial chaperone DnaK [20] or in the 'superoligomerization' of the small heat shock proteins upon heat shock [21]. In addition to the homooligomerization features, ERp29 may also interact with other polypeptides, as judged by its co-immunoprecipitation with a number of ER proteins (Fig. 3B). One of them was previously identified as BiP/GRP78 [8], which raises the intriguing possibility of the putative cooperation of ERp29 with BiP/GRP78 or other chaperones in the folding/refolding of secretory proteins in the ER.

ERp29 shares limited similarity with the group of protein disulfide isomerases from various organisms including plants, amoebae, a recently discovered protein from *Drosophila* with an unknown function [22], and to a lesser extent with the mammalian PDIs. The latter enzymes have a modular structure [23] and consist of two homologous thioredoxin domains (a and a′) containing an active site motif -Cys-Gly-His-Cys- involved in thiol-disulfide reactions, two intermediate domains (b and b′) lacking clearly assigned function and a C-terminal domain believed to be important for substrate binding [24]. Recent NMR studies on the structure of PDI domains have shown that, despite the lack of homology between segments a and b, each of them adopts a global fold very similar to that of a bacterial thioredoxin and the same structure was predicted for domains a′ and b′ [25,26]. Thus, it was suggested that the PDI molecule essentially consists of linked active and inactive thioredoxin-like modules, the structure that may have been evolved by means of partial gene duplication or shuffling of a common thioredoxin ancestral gene [26].

The sequence similarity between ERp29 and PDI (26% identity over a region of 111 amino acids) is detected in the N-termini of both proteins (Fig. 4), i.e. between the N-terminal segment of ERp29, comprising amino acids 1–126, and the a domain of PDI, although the active thioredoxin motif is absent in ERp29. Interestingly, alignment of ERp29 and ERp31 (a human analogue of ERp29; unpublished, GenBank accession number X94910) sequences [10] indicates a very strong conservation of the N-terminal domains of the human and rat proteins, thus highlighting its potential functional importance.

When ERp29 sequence was scrutinized by the secondary structure prediction and fold recognition methods [15,16], the sequence similarity between the N-domain of ERp29 and the a domain of PDI was augmented by the prediction of alike α/β-type secondary structure (Fig. 4) and the same domain of ERp29 was predicted to adopt a thioredoxin-like global fold similar to the a and b domains of PDI. PDB database entry 1mek (structure of the a domain of PDI resolved by NMR) was the only one with a Z-score significantly over the confidence threshold needed to confirm the fold recognition (prediction results are available at http://www.doembi.ucla.edu/people/frsvr/preds/18329ERp29/18329ERp29.html).

The same domain of ERp29 also shares 34% identity over a stretch of 56 amino acids with the b domain of PDI which may reflect the early divergence of ERp29 from the ancestral PDI gene when the a and b domains were still closely related. Despite the decrease in sequence similarity between all three domains (a/b of PDI and N-terminal of ERp29) in the course of evolution, their thioredoxin-like conformation remained remarkably intact. A similar conservation of the three-dimensional structures was reported for the distantly related proteins where the amino acid identity level appeared to be almost random [27].

Darby et al. [28], analyzing the activity of various PDI constructs with reshuffled domain structures, found that full catalytic activity requires cooperative involvement of multiple PDI domains, and b/b′ domains are of particular importance. It was suggested that these internal repeats enhance and diversify substrate binding capabilities of PDI as multiple thioredoxin modules may provide multiple sites of contact with substrate proteins [29–31].

Considering the similarities in sequence and conformation of the a and b domains of PDI and the N-terminal segment of ERp2 and also the advantages of multidomain composition with intrinsic homology, we hypothesize that dimerized ERp29 is essentially a variant of a modular molecule similar to PDI with two thioredoxin-like subunits placed close to each other – the structure that apparently favors efficient protein

binding. A similar oligomeric conformation is characteristic also of some thioredoxin-like proteins with different functions, such as glutathione *S*-transferase (dimer) and glutathione peroxidase (tetramer) [32]. Extensive interactions with diverse protein substrates are vital not only for the thiol isomerase activity of PDI but also for the general chaperone function, that is for binding to and refolding the misfolded proteins. The latter function is supported by the finding that PDI may fold proteins without internal disulfide bonds [33,34] and also by its integration in the prolyl hydroxylase [35] and triacylglycerol transfer protein [36] complexes, where PDI does not appear to have a redox activity, but instead has been suggested to function as an internal folding factor stabilizing the conformation of the complex.

Based on these observations we predict that ERp29, a dimeric protein with a thioredoxin-like structure, which probably lost its redox activity in the course of evolution, may still retain extensive protein-protein interacting features characteristic of molecular chaperones or some related enzymes. It seems that this assumption is consistent with other essential traits of ERp29, such as stress inducibility, interaction with BiP/GRP78, widespread expression and ER targeting. However, the purpose of the putative protein binding capabilities of ERp29 still remains to be established as no characteristic motifs, indicating possible function, such as active cysteine sites are present in its sequence.

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