Functional roles and efficiencies of the thioredoxin boxes of calcium-binding proteins 1 and 2 in protein folding

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The rat luminal endoplasmic-recticulum calcium-binding proteins 1 and 2 (CaBP1 and CaBP2 respectively) are members of the protein disulphide-isomerase (PDI) family. They contain two and three thioredoxin boxes (**Cys**-Gly-His-**Cys**) respectively and, like PDI, may be involved in the folding of nascent proteins. We demonstrate here that CaBP1, similar to PDI and CaBP2, can complement the lethal phenotype of the disrupted *Saccharomyces cerevisiae* PDI gene, provided that the natural C-terminal Lys-Asp-Glu-Leu sequence is replaced by His-Asp-Glu-Leu. Both the *in vitro* RNase AIII-re-activation assays and *in vivo* pro-(carboxypeptidase Y) processing assays using CaBP1 and CaBP2 thioredoxin (trx)-box mutants revealed that, whereas the three trx boxes in CaBP2 seem to be functionally equivalent, the first trx box of CaBP1 is significantly more active than the second trx box. Furthermore, only about 65% re-activation of denatured reduced RNase AIII could be obtained with CaBP1 or CaBP2 compared with PDI, and the yield of PDI-catalysed reactions was significantly reduced in the presence of either CaBP1 or CaBP2. In contrast with PDI, neither CaBP1 nor CaBP2 could catalyse the renaturation of denatured glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which is a redox-independent process, and neither protein had any effect on the PDI-catalysed refolding of GAPDH. Furthermore, although PDI can bind peptides via its b′ domain, a property it shares with PDIp, the pancreas-specific PDI homologue, and although PDI can bind malfolded proteins such as 'scrambled' ribonuclease, no such interactions could be detected for CaBP2. We conclude that: (1) both CaBP2 and CaBP1 lack peptide-binding activity for GAPDH attributed to the C-terminal region of the a′ domain of PDI; (2) CaBP2 lacks the general peptide-binding activity attributed to the b′ domain of PDI; (3) interaction of CaBP2 with substrate (RNase AIII) is different from that of PDI and substrate; and (4) both CaBP2 and CaBP1 may promote oxidative folding by different kinetic pathways.

Key words: endoplasmic reticulum, ERp72, oxidase, P5, protein disulphide-isomerase.

INTRODUCTION

Protein disulphide-isomerase (PDI) is a soluble luminal protein of the endoplasmic reticulum (ER) [1] that can catalyse the formation and isomerization of disulphide bonds in target proteins, and has been implicated in protein folding (for a review, see [2]). The protein contains two copies of the conserved thioredoxin (trx) box Cys-Gly-His-Cys that is also found in several other PDI-like proteins existing in the ER. The trx box occurs in a domain, the thioredoxin fold, which is highly conserved between PDI-like proteins and thioredoxin. The cysteines within the trx boxes are redox active. In addition to these two redox-active trx-fold domains (designated a and a′), PDI has two redox-inactive trx-fold domains termed b and b′ [2]. The major binding site for peptides is distinct from the trx boxes. Primarily the b′ domain has been implicated, although other domains may participate as well [3–6]. For the interaction of PDI with denatured glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the C-terminal portion of the a′ domain seems to be of paramount importance [7].

The essential function of PDI in yeast resides mainly in the isomerase activity of its trx boxes. It has been demonstrated that only the first (i.e. N-terminal) cysteine residues in each trx box are necessary for the isomerase activity of PDI [8–11], and that the lethal yeast phenotype PDI1Δ [12] can be alleviated by overexpression of mammalian PDI, mammalian ERp72/CaBP2 [12] or yeast MPD2 [13] (MPD is derived from multicopy suppressor gene of the *pdi1* deletion). In fact, even trx can rescue PDI1Δ yeast, as long as the relatively reducing Cys-Gly-Pro-Cys trx- box sequence is replaced by one with a higher redox potential, e.g. the PDI-like Cys-Gly-His-Cys tetrapeptide [14,15].

PDI clearly possesses chaperone activities *in vitro* [16,17] and in intact cells [18–20] which are independent of intact trx boxes, in particular of the N-terminal cysteine residues in these boxes.

The relative efficiencies of the N- and C-termini of either active trx box in protein folding has been examined in different assay systems [10,21,22], showing the human a domain of PDI to be more active than the a′ domain. Interestingly, in yeast the inverse is true, with a greater redox activity being attributed to the a′ domain.

PDI is only one protein out of a large family of proteins with trx boxes residing in the ER [2], but the roles of these PDI-family members is poorly understood. We have studied the two PDI-like proteins CaBP1 and CaBP2 [23–25] (CaBP1 is the rat homologue of human and hamster P5 and CaBP2 is the rat homologue of human ERp72). These relatively abundant ER luminal proteins contain three and five trx domains respectively, of which two in CaBP1 and three in CaBP2 possess active trx

Abbreviations used: CaBP, calcium-binding protein (CaBP1 is the rat homologue of human and hamster P5, and CaBP2 is the rat homologue of human ERp72); CPY, carboxypeptidase Y; ER, endoplasmic reticulum; 5-FOA, 5-fluoro-orotic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PDI, protein disulphide-isomerase; PDIp, pancreas-specific PDI homologue; sc, scrambled; trx, thioredoxin; GST, glutathione S-transferase; DSG, disuccinimidyl glutarate; WT, wild-type.

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boxes [2]. Both proteins have been shown to catalyse refolding of denatured RNase AIII [26] and both catalyse reduction of the disulphide bonds of insulin [24,27].

In the present study we compared the effects of wild-type (WT) CaBP1 and CaBP2 and of CaBP1 and CaBP2 with different mutations in their trx boxes in three different assay systems: (1) renaturation of reduced, denatured RNase AIII and refolding of 'scrambled' RNase AIII (scRNase AIII), (2) suppression of a PDI1Δ yeast mutant lethal phenotype and (3) processing of the carboxypeptidase Y (CPY) precursor in yeast.

We also compared the trx-box-independent ability of (1) CaBP1, CaBP2 and PDI to catalyse refolding of a denatured substrate, GAPDH, which lacks disulphide bonds, (2) CaBP2 and PDI to bind a peptide substrate, Δ-somatostatin, and (3) CaBP2 and PDI to interact with an oxidized, malfolded protein substrate (RNase AIII).

We found that, in all redox-dependent assay systems, CaBP1 and CaBP2 can functionally replace PDI, albeit with different efficiencies. These effects depend on the presence of intact trx boxes or boxes which contain at least the N-terminal cysteine residues in their trx boxes. Although each single trx box in PDI, CaBP1 and CaBP2 possesses activities in the various test systems, the potency of the boxes of a given protein are not equivalent, as shown for PDI and CaBP1, where the first (N-terminal) trx box is more active than the second one.

However, neither CaBP1 nor CaBP2 can functionally replace the redox-independent chaperone activity of PDI in the refolding of denatured GAPDH, and CaBP2 also seems to lack the peptide-binding activity of the PDI b′ domain and also does not bind a denatured, but oxidized, substrate (scRNase AIII).

Thus we conclude that, although CaBP1 and CaBP2 can functionally replace PDI in its redox/isomerase roles, they seem to differ from PDI in lacking general chaperone and peptide-binding activities.

EXPERIMENTAL

Construction of *Saccharomyces cerevisiae* strains

Strain BK203-15B, a haploid yeast strain carrying a disruption of the PDI gene, was constructed as follows. One copy of the yeast PDI gene in diploid strain BK201 (genotype *a*/α; *leu2*/*leu2*; *his3*/*his3*; *trp1*/*trp1*; *ura3*/*ura3*) was disrupted via a one-step gene disruption using the 4.0 kb *Sst*I fragment of plasmid PSKpdi::LEU (see below). The genotype of the resulting strain BK 203 was confirmed by tetrad analysis after sporulation and through amplification of the PDI 'region' by PCR using chromosomal DNA and oligonucleotides 5′-CACAGATCTAGACGAATGACATCTTGTGTATG-3′ and 5′-GATTGCGCATGCTCGAGCTCGCAGTAAAGCAAGTGTCC-3′. This strain was then transformed with plasmid pWBK-PDI. Transformants were sporulated and spore colonies analysed. Strain BK203-15B (genotype: *mat a*; *leu2*, *his3*, *trp1*, *ura3*, *pdi*::*LEU2*; *pWBK-PDI*) was chosen as a tester strain for the complementation assay.

Construction of PDI plasmids

The *S. cerevisiae* PDI gene, including the promoter and other control regions, was amplified by PCR using oligonucleotides 5′-CACAGATCTAGACGAATGACATCTTGTGTATG-3′ and 5′-GATTGCGCATGCTCGAGCTCGCAGTAAAGCAAGTGTCCAC-3′. The amplified PDI gene was used for various plasmid constructions. For construction of the centromeric plasmid pWBK-PDI, the PDI gene was cloned as a 2.4 kb *Xba*I/*Sst*I fragment into the *Xba*I/*Sst*I restriction sites of vector pWBK3 [28]. For construction of plasmid pSK-pdi::LEU, which was used to create the PDI disruption strain, the PDI gene was first cloned on a 2.4 kb *Xba*I/*Xho*I restriction fragment into the *Xba*I/*Xho*I restriction sites of the vector pBSK(−) (Stratagene) to obtain plasmid pSK-PDI. The *LEU2* gene was cloned on a 2.4 kb *Bgl*II/*Sal*I fragment into the *Bgl*II/*Hin*dIII restriction sites of pSK-PDI. This resulted in plasmid pSK-pdi::LEU, in which the internal 280 bp *Hin*dIII- and 505 bp *Hin*dIII/*Bgl*II restriction fragments of PDI were replaced by the *LEU2* gene.

Construction of trx-box mutants of PDI

For the construction of the trx-box mutants and subsequent cloning into the yeast expression vector pDS2 [12] a *Bam*HI site was introduced using site-directed mutagenesis immediately adjacent to the first ATG codon of PDI, digestion of the resulting pSK-PDI-Bam plasmid with *Bam*HI and *Xba*I, and subsequent ligation of the filled-in restriction sites resulting in plasmid pSK-PDIex, in which all the 5′-control regions had been removed. Using this plasmid the various trx-box mutations (see Figure 1 below) were introduced by site-directed mutagenesis

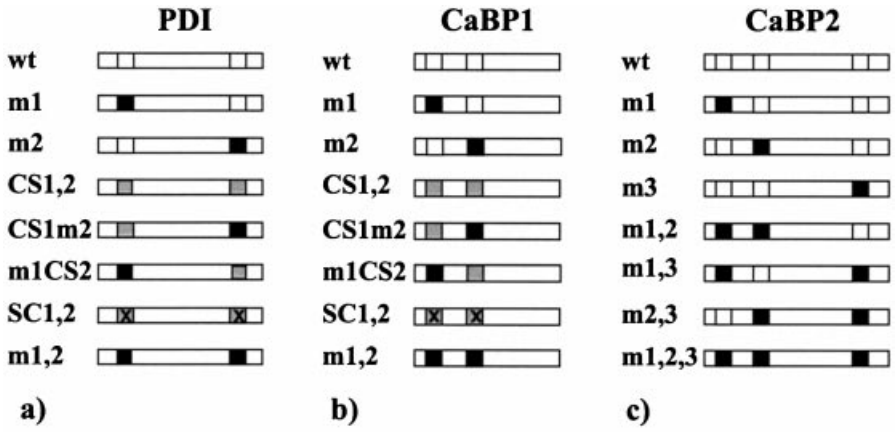

Figure 1 Recombinant proteins used in the present study

Variants of PDI, CaBP1 and CaBP2 are shown in (**a**), (**b**) and (**c**) respectively. The trx boxes have the following sequences and shadings: white boxes, wt (≡ WT; Cys-Gly-His-Cys); black boxes, m (Ser-Gly-His-Ser); grey boxes, CS (Cys-Gly-His-Ser); grey boxes with black crosses, SC (Ser-Gly-His-Cys). Numbers indicate which of the trx boxes are concerned.

[29]. All mutations and the integrity of the remaining DNA sequence were verified by nucleotide sequence analysis. To analyse the effect of the mutations on the growth of yeast cells the mutant genes were cloned as a *Bam*HI/*Sal*I restriction fragment into the *Bam*HI/*Sal*I restriction sites of the yeast expression vector pDS2. The genes are here under the control of the yeast *gal1* promotor.

Construction of CaBP1 plasmids

The open reading frame of the CaBP1 gene was amplified from pCMV2-CaBP1 [24] by PCR and cloned into plasmid pBSK(−) as a *Bam*HI/*Sal*I fragment. Two versions of the CaBP1 gene were constructed which both contained a *Bam*HI restriction site immediately before the start ATG codon. However, in one copy the sequence coding for the rat ER retrieval signal (-KEEL) was left intact (pSK-rCaBP1), whereas in the other (pSK-yCaBP1) the retention signal was changed to -HDEL, the yeast retention signal. For the purpose of creating shuffling mutants between different vectors, pSK-yCaBP1 was further modified to plasmid pSK-CaBP1 by introduction of a *Sac*I restriction site at nucleotide positions 66–71, which did not change the amino acid sequence.

The different trx-box mutations (see Figure 1 below) were introduced by site-directed mutagenesis [29] using the plasmid pSK-CaBP1 as the template. All mutations and the integrity of the remaining DNA sequence were verified by nucleotide sequence analysis.

For expression of CaBP1 in yeast the 1.3 kb *Bam*HI/*Sal*I fragment of pSK-CaBP1 containing the WT sequence was cloned into the *Bam*HI/*Sal*I-digested pDS2 yeast expression vector. The various plasmids containing the trx-box mutants were created by exchanging the internal 1.2 kb *Sac*I fragment with the respective ones from pSK-CaBP1 mutant clones.

For protein expression in *Escherichia coli*, a *Bam*HI site was introduced into pSK-rCaBP1 immediately before the start of the sequence coding for the mature protein, together with the *Sac*I site at the same position as in pSK-CaBP1. The resulting vector (pSK-mCaBP1) contained only the sequence coding for the mature protein. To produce glutathione S-transferase (GST)–CaBP1 fusion proteins, the 1.25 kb *Bam*HI/*Sal*I-fragment was then cloned into the pGEX-4T vector (Pharmacia) to give pGEX-mCaBP1. The mutant genes were again introduced by replacing the 1.2 kb *Sac*I restriction fragment.

Construction of CaBP2 plasmids

For CaBP2, the following plasmid constructs were made: pSK-rCaBP2, the rat gene amplified by PCR and cloned via *Bam*HI and *Xho*I; pSK-yCaBP2, coding for CaBP2 with His-Asp-Glu-Leu as the yeast retrieval signal; pSK-CaBP2, a CaBP2 construct coding for His-Asp-Glu-Leu as retrieval signal and an additional *Mlu*I restriction site at position 89–94 of the coding sequence; pDS-CaBP2, CaBP2 (His-Asp-Glu-Leu *Mlu*I type) cloned via *Bam*HI/*Xho*I into *Bam*HI/*Sal*I of the yeast expression vector pDS2; pSK-mCaBP2, contains the sequence coding for mature CaBP2 containing a *Bam*HI site immediately before the start of the sequence coding for the mature protein, the *Mlu*I site at positions 89–94, and coding for the rat retrieval signal Lys-Asp-Glu-Leu; pGEX-mCaBP2, a construct to produce CaBP2 (mature) protein in *E. coli*. All pSK-CaBP2, pDS-CaBP2 and pGEX-mCaBP2 trx-box mutants (see Figure 1) were constructed similarly to the CaBP1 mutants.

For expression in *E. coli* of recombinant CaBP2 for use in the cross-linking experiments, cDNA coding for full-length CaBP2, without the signal sequence, was used for the construction of the vector based on pET12. Similar vector constructs were generated to produce full-length human PDI [5].

Construction of the PDIp plasmid

The expression vector for PDIp (the pancreas-specific PDI homologue) was constructed corresponding to Glu^{9}–Leu^{511} of the full-length sequence of PDIp [30]. The DNA insert was prepared by PCR from the original PDIp construct described by DeSilva et al. [30], using primers that allowed insertion of a *Nco*I site at the N-terminus and a *Sal*I site at the C-terminus. The insert was cloned between the *Nco*I and *Sal*I site of pET23d.

Determination of RNase refolding

Denaturation and reduction of RNase AIII was carried out essentially as previously described [26]. Refolding assays and calculation of active RNase AIII were performed as described by Lyles and Gilbert [31], with the correction introduced by Rupp et al. [26]. Renaturation of 5 μM RNase was monitored in the presence of 1 mM GSH, 0.2 mM GSSG, and 4.5 mM cCMP over a period of 40 min with measurements taken every 30 s. The maximum yield of re-activated RNase could differ somewhat on a batch-to-batch basis, and was always verified by control experiments with PDI.

For the preparation of scRNase AIII, 25 mg of denatured, reduced RNase was incubated in 5 ml of scrambling buffer (6 M guanidinium chloride/100 mM Tris/HCl/2 mM EDTA, pH 8.0) for 3 days in the dark under aeration and constant stirring. The scRNase was then dialysed against 0.1 % acetic acid and kept at 0 °C. Free thiol groups were titrated with Ellman's reagent and were determined to be less than 3 % of all cysteine residues in the RNase.

Renaturation of scRNase AIII was carried out and monitored similar to renaturation of reduced, denatured RNase.

The comparison between the refolding and renaturing activities of the recombinant WT and mutant proteins required that the amount of protein added to the test systems was identical. This was done by (1) calculating the protein concentration from A_{280} [32], (2) measuring the protein concentration as decribed by Bradford [33], and (3) by performing for each protein calibration curves by running SDS/PAGE with different protein concentrations and staining with Coomassie Blue.

Complementation of PDI disruption

For complementation, yeast strain BK203-15B was used, which carries a disruption of the chromosomal PDI gene. Since this disruption is lethal, a functional copy of the PDI gene is provided in this strain on the centromeric plasmid pWBK-PDI carrying also the *URA3* gene. Strain BK203-15B was then transformed with 2 μg of plasmid pDS2 containing different alleles of PDI, CaBP1, and CaBP2 under control of the GAL1 promoter and as a selectable marker the *HIS3* gene. After initial selection on Synthetic, Complete medium with agar ('SC-agar') plates with 2 % (w/v) glucose lacking histidine, transformants were transferred to histidine-free plates containing 2 % galactose to express the gene to be analysed. After growth on plates for 24–48 h, yeast cells were stamped to plates containing 5-fluoro-orotic acid (5-FOA; [34]), a compound toxic to cells containing the *URA3*-gene product (e.g. cells transfected with the pWBK-PDI plasmid). This leads to exclusive growth of cells that have lost plasmid pWBK-PDI and contain an allele of PDI, CaBP1 or CaBP2 respectively, which is able to replace the PDI gene functionally.

In order to verify the ability of the alleles of PDI, CaBP1 or CaBP2 to complement the PDI gene disruption at lower expression levels, growth of cells lacking plasmid pWBK-PDI on media containing glucose was also monitored (see Figure 1).

Analysis of CPY processing

For the analysis of effects of PDI, CaBP1 and CaBP2 and the respective mutants on CPY processing, pulse–chase experiments were carried out as described in [35].

Variants of strain BK 203-15B were used lacking the centromeric plasmid pWBK-PDI, but bearing the genes to be tested on the expression plasmid pDS. These strains were grown overnight in synthetic yeast medium lacking histidine (selection of the plasmid), leucine (selection for PDI disruption), cysteine and methionine with 1% galactose as the carbon source (SCgal-HLCM). A total of 10^8 cells were diluted into 25 ml of fresh growth medium and incubated for 1.5 h at 30 °C with shaking. The cultures were then centrifuged at 1000 ***g*** and the cells resuspended in 500 μl of SCgal-HLCM medium containing 200 μCi of Express label (NEN; cysteine, 235 Ci/mmol; methionine, 940 Ci/mmol). After 15 min growth at 30 °C with shaking, 1 ml of chase-mix (SCgal-HLC/1 mM NH_4SO_4/0.2 mM L-cysteine/0.2 mM L-methionine) was added. Aliquots (300 μl each) were drawn after 0, 5, 15 and 60 min. The cells were pelleted, washed with PBS/10 mM NaN_3 and resuspended in 50 μl of lysis buffer [PBS/1% SDS/0.02% PMSF/10 mM benzamidine/1.75 μg/ml *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane ('E-64')/2 μg/ml aprotinin/1 μg/ml chymostatin/1μg/ml leupeptin/1μg/ml antipain/1μg/ml pepstatin/1 mM phenanthroline]. A 150 μl portion of glass beads (diameter $\approx$ 500 μm) were added, and, after extensive vortex-mixing, extracts were frozen in liquid N_2 and stored at -20 °C until use.

For immunoprecipitation of CPY or its precursors extracts were thawed on ice, vortex-mixed for five 1 min bursts with cooling intervals at 0 °C, followed by heating at 95 °C for 3 min. Glass beads and insoluble material were removed by centrifugation and the supernatant preserved. The glass beads were washed twice with IP buffer (1% Triton X-100/0.2% SDS/150 mM NaCl/5 mM EDTA/50 mM Tris/HCl, pH 7.4) and the washing solutions pooled with the first supernatant. Following pre-incubation with 10 μl of Protein A–Sepharose for 30 min at 20 °C, Protein A–Sepharose was removed by centrifugation and the supernatants were incubated with another 20 μl of Protein A–Sepharose plus polyclonal antibodies against CPY (obtained from M. Benli, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany) overnight at 4 °C. Following sedimentation by centrifugation the Protein A–Sepharose pellets were washed once with respectively 1 ml of IP buffer containing 2 M urea, IP buffer containing additional 500 mM NaCl, IP buffer and IP buffer lacking SDS and Triton X-100. The Protein A–Sepharose pellets were finally resuspended in Laemmli gel sample buffer lacking 2-mercaptoethanol, heated for 2 min at 95 °C, and subjected to SDS/PAGE. The dried gels were analysed for radioactivity using a Bio-Rad Phospho-imager GS42.

Production of recombinant rat CaBP1, CaBP2 and human PDIp in *E. coli*

Typically a 1-litre culture of strain BL21 (*F′*, *dcm*, *ompT*, *hsdS*(r_B^-,m_B^-), *gal*; Stratagene) bearing the respective pGEX plasmids (CaBP1 or CaBP2) was grown at 30 °C to an attenuance (D_{600}) of 0.8. Protein expression was induced by the addition of 0.5 mM isopropyl β-D-thiogalactoside and the culture further incubated for 2 h at 30 °C. Cells were harvested and lysed by sonication. Cell debris was removed by centrifugation at 40 000 ***g*** for 1 h. The GST-fusion proteins were purified by affinity chromatography on GSH–agarose (Sigma). The fusion proteins were eluted from the column with 10 mM GSH and digested with thrombin (10 units/mg of fusion protein). GST and proteolytic breakdown products of the CaBP proteins were subsequently removed by gel filtration over Superdex-200/16. Fractions containing the purified cleaved proteins were immediately adjusted to 80% saturation by slowly adding solid $(NH_4)_2SO_4$ at 4 °C. After 2 h the protein was precipitated by centrifugation for 30 min at 14000 ***g*** and 4 °C. The samples were stored at 4 °C. For use, proteins were suspended in the appropriate assay buffer and $(NH_4)_2SO_4$ was removed by gel filtration over NAP-5 desalting columns (Amersham) that had been equilibrated with the same buffer. The proteins were stored for up to 48 h at 0 °C. Before use all proteins were subjected to SDS/PAGE in order to control for breakdown products.

For cross-linking assays with Δ-somatostatin and scRNase AIII, production of CaBP2 from the pET12 construct and PDIp from the pET23d construct was carried out in *E. coli* strain BL21 (DE3), which also contained the pLysS plasmid to control leakthrough expression. Cell extracts from *E. coli* were prepared by repeated freezing and thawing and were used without any further purification.

Preparation of PDI, CaBP1/P5 and CaBP2/ERp72 from bovine liver

PDI used in the present study was purified from bovine liver as described by Rowling et al. [36]. CaBP1 and CaBP2 were prepared essentially as described by Fleischer and Kervina [37] and Nguyen Van and colleagues [23].

GAPDH-renaturation assay with PDI, CaBP2 and CaBP1

PDI can promote the refolding of denatured GAPDH (Boehringer). This has been proposed [16] to indicate the ability of PDI to promote correct folding of a substrate without intermediate disulphide-bond formation and isomerization. GAPDH has only one cysteine residue per monomer, and the reducing conditions employed for the assay suffice for retention of this residue in the reduced state.

The GAPDH refolding assay was performed as described by Cai et al. [16] using PDI, CaBP1/P5 and CaBP2/ERp72 purified from bovine liver. The final concentrations of the three proteins in the assay ranged from 0 to 60 μM, while that of the substrate was kept at 2.8 μM. Briefly, GAPDH, denatured in guanidinium chloride overnight at 4 °C, was renatured by placing portions into a solution containing buffer [0.1 M KH_2PO_4 (pH 7.5)/5 mM dithiothreitol/1 mM EDTA] and 0, 1.5, 7, 15, 30 and 60 μM respectively of PDI, CaBP2 or CaBP1. The final concentration of GAPDH was 2.8 μM in all cases, and the reaction volume was 100 μl. The tubes were incubated at 4 °C for 30 min, then at 20 °C for 3 h. Thereafter, 10 μl of the reaction mixture were taken and the activity of properly refolded GAPDH measured as the production of NADH from NAD^+ (Boehringer) at 340 nm in a Shimadzu UV 160A spectrophotometer, using D,L-glyceraldehyde 3-phosphate (D,L-GAP diethylacetal; Sigma) and sodium arsenate as described elsewhere [16].

Binding of Δ-somatostatin and scRNase

Labelling of Δ-somatostatin (Ala-Gly-Ser-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Ser, synthesized as described pre-

viously for other peptides [38]) with [^{125}I]Bolton–Hunter reagent {*N*-succinimidyl-3-(4-hydroxy-5-[^{125}I]iodophenyl)propionate} was performed as described by the manufacturer (Amersham). After precipitation with trichloroacetic acid, the radiolabelled Δ-somatostatin was dissolved in distilled water. Labelled Δ-somatostatin or scRNase (Sigma) was added to buffer A (100 mM NaCl/25 mM KCl/100 mM phosphate buffer, pH 7.5), containing the crude cell extracts. The samples (10 μl) were incubated for 10 min on ice before cross-linking.

Cross-linking was performed using the homobifunctional cross-linking reagent disuccinimidyl glutarate (DSG; Sigma) [39]. The samples were supplied with 1/10 volume of cross-linking solution (10 mM DSG in buffer A). The reaction was carried out for 60 min at 0 °C. Cross-linking was stopped by the addition of SDS/PAGE sample buffer [40,41].

The samples were subjected to electrophoresis in SDS/12.5 %-(w/v)-polyacrylamide gels with subsequent autoradiography. Cross-linking products containing scRNase were detected using an anti-CaBP2 and anti-PDI antibody respectively, after electrotransfer on to PVDF membranes. Detection was performed using the enhanced-chemiluminescence (ECL®) kit from Amersham.

RESULTS

We have shown previously that, similarly to PDI, CaBP1 and CaBP2 isolated from rat liver are able to enhance refolding of RNase AIII as well as of a Fab fragment directed against creatine phosphokinase [26]. Oxidative refolding of RNase AIII by PDI [11] involves an oxidoreductase as well as an isomerase activity, which depend on the presence of active trx boxes. A detailed analysis of the two trx boxes (**Cys**-Gly-His-**Cys**) of PDI revealed that they are not equivalent. Whereas inactivation of the first box affects k_{cat}, inactivation of the second box leads to increased K_m [42]. Furthermore, it was shown that the oxidoreductase activity required the presence of both cysteine residues of a trx box, whereas only the first (i.e. N-terminal) cysteine was necessary for the isomerase reaction [8–11]. This has not been examined yet for CaBP1 or CaBP2. Therefore we constructed mutants of CaBP1 where either of the two trx boxes (CaBP1 m1 and CaBP1 m2) or both (CaBP1 m1,2) were inactivated through the replacement of the cysteine residues by serine residues. A second set of CaBP1 mutants was made in which only one of the cysteine residues in the trx boxes was replaced (CaBP1 CS, CaBP1 CS1m2 and CaBP1 m1CS2; see also Figure 1 for a description of the mutants).

The constructed CaBP2 mutants contained either one (CaBP2 m1, CaBP2 m2, and CaBP2 m3), two (CaBP2 m1,2, CaBP2 m1,3 and CaBP2 m2,3) or three (CaBP2 m1,2,3) completely inactivated boxes (see Figures 1a–1c).

Refolding of RNase AIII

Refolding of denatured, reduced RNase by CaBP1, CaBP2 and their mutants

The different mutants of CaBP1 and CaBP2 were expressed as GST-fusion proteins in *E. coli*. In these constructs the GST protein was followed by the amino acid sequence of the mature rat protein (i.e. protein without signal sequence) separated by the recognition sequence for the thrombin protease. After affinity purification of the fusion proteins via GSH–agarose affinity chromatography, digestion with thrombin and gel filtration, the different CaBP1 and CaBP2 variants were tested for their ability to refold denatured and reduced RNase AIII (see the Experimental section).

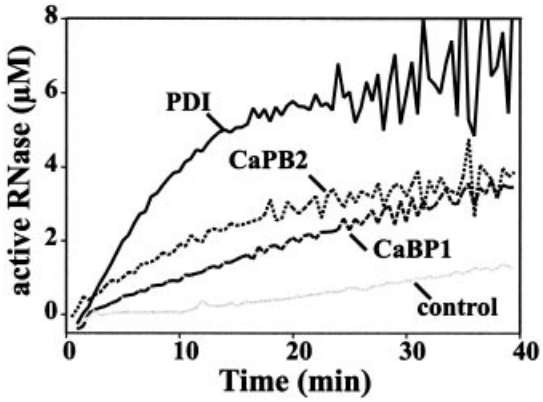

Figure 2 Re-activation of denatured RNase by PDI proteins

Plot of the concentration of active RNase AIII as a function of refolding time. In the presence of 5 μM PDI, practically all of the RNase activity present before denaturation was recovered. The activities of recombinant CaBP2 and CaBP1 (5 μM each) were significantly lower than that of PDI, about 60%. As a negative control, denatured RNase treated identically but without 'foldase' was used (foldase means chaperone activity and/or redox/isomerase activity, i.e. any activity that helps fold a protein to its native state).

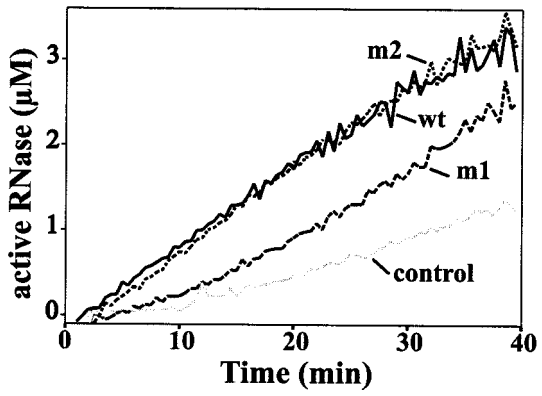

Figure 3 Effect of trx-box inactivation in CaBP1 on the re-activation of denatured reduced RNase

m1 and m2 indicate that the first or second trx box respectively has been inactivated by mutagenesis, by converting the active-site Cys-Gly-His-Cys into Ser-Gly-His-Ser. Whereas the m1 mutant clearly displays reduced activity as compared with WT, the mutant with the first trx box intact (m2) retains WT activity. All CaBP1 proteins were used at 2.8 μM. As a negative control, denatured RNase treated identically but without foldase was used.

Both recombinant CaBP2 as well as recombinant CaBP1 promoted the folding of RNase AIII (Figure 2). The velocity with which active RNase AIII was produced increased with the concentration of the two proteins, a maximal effect occurring at 10 μM. However, in comparison with PDI (isolated from bovine liver), CaBP1 and CaBP2 were less active. Moreover, in the presence of CaBP1 and CaBP2 only about 60 % of the RNase AIII became re-activated, whereas with PDI 100 % of the initial RNase activity was recovered (Figure 2).

Variants of CaBP1 and CaBP2 in which all cysteine residues present in the trx boxes had been replaced by serine residues (CaBP1 m1,2 and CaBP2 m1,2,3) no longer enhanced the refolding of denatured, reduced RNase AIII (Figures 3 and 4). However, refolding of RNase AIII was still enhanced by CaBP2 or CaBP1 mutants in which at least one trx box had been left

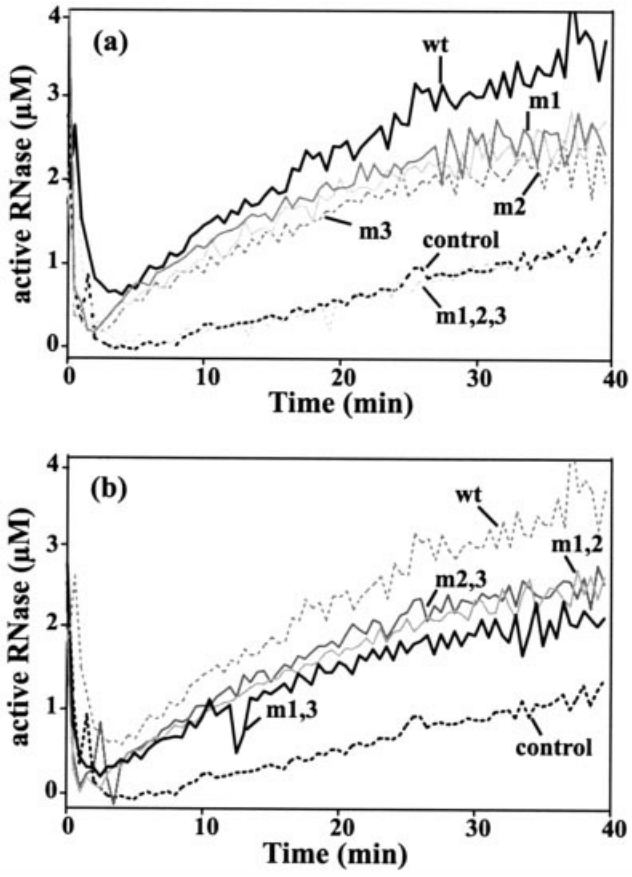

Figure 4 Effect of trx-box inactivation in CaBP2 on the re-activation of denatured reduced RNase

m1, m2 and m3 indicate that the first, second or third trx box respectively has been inactivated by mutagenesis, by converting the active-site Cys-Gly-His-Cys into Ser-Gly-His-Ser. Single (**a**) and double (**b**) trx-box mutants clearly display reduced activity as compared with WT CaBP2 (all proteins at 2 μM). In contrast with CaBP1 mutants, trx boxes in CaBP2 display similar activities. As a negative control, denatured RNase treated identically but with either inactive foldase (m1,2,3) (**a**) or without foldase was used.

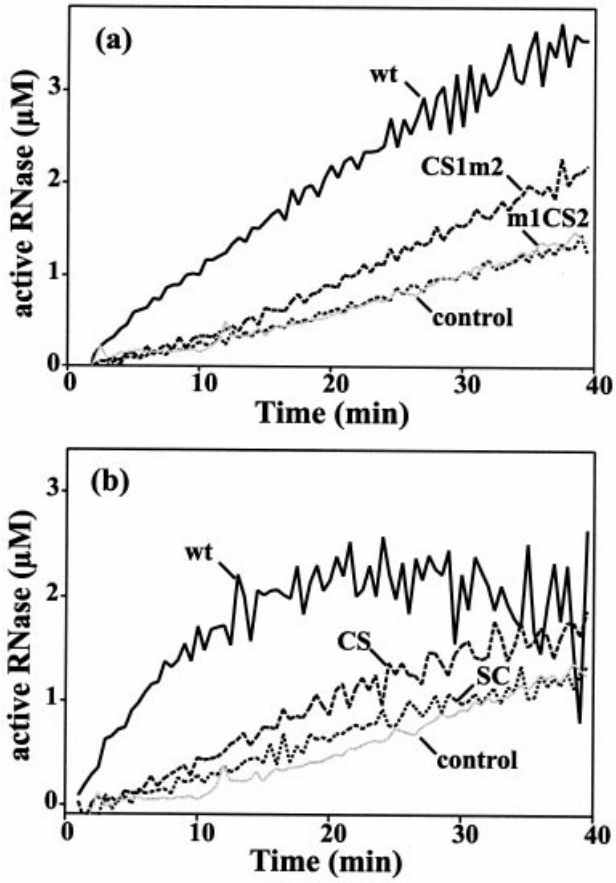

Figure 5 Effect of mutants of CaBP1 on the re-activation of denatured reduced RNase

See the legend to Figure 1 for abbreviations. (**a**) Although CaBP1 CS1m2 (2.8 μM) still retains activity in renaturation of RNase AIII, m1CS2 (2.8 μM) completely lacks activity. Thus the first trx box contributes the major refolding activity. (**b**) As expected, CaBP1 with both trx boxes mutated to Ser-Gly-His-Cys (SC) displays very low to no significant activity. In contrast, the CS mutant retains activity similar to CaBP1 m1 (see Figure 3). The final concentration of each CaBP1 protein was 10 μM. As a negative control, denatured RNase treated identically but without foldase was used. Note that the maximum yield of reactivated RNase in the presence of CaBP1 WT in (**b**) is lower than that obtained in (**a**). This is entirely due to batch-to-batch differences in the RNase preparation. A similar decrease was also observed for a PDI control reaction (results not shown).

intact (Figures 3 and 4). This demonstrates that the refolding activities of these two PDI family members require their trx boxes.

Interestingly, the two trx boxes of CaBP1 contributed to the overall activity to different extents (Figure 3). The replacement of both cysteine residues in the second box by serine residues (CaBP1 m2) had no detectable effect on the velocity of the refolding as compared with the recombinant WT CaBP1. This was so at all concentrations of CaBP1 tested (1.4–20 μM). On the other hand, exclusive inactivation of the first box (CaBP1 m1) greatly reduced the activity of CaBP1 (Figure 3). The CaBP1 mutant in which the C-terminal cysteine residues of both boxes had been replaced by serine residues (CaBP1 CS, Figure 5b) showed a reduced activity that was similar to that of CaBP1 m1 (Figure 3). A difference between the two boxes of CaBP1 was also observed when, in addition to the replacement of both cysteine residues of one box by serine residues, the second (i.e. C-terminal) cysteine residue of the other box was replaced by serine (CaBP1 m1CS2 and CaBP1 CS1m2) (Figure 5). Whereas CaBP1 with no cysteine residues in the second box and only the N-terminal cysteine residue in the first box (CaBP1 CS1m2) still enhanced renaturation of RNase AIII, we could no longer detect such an activity with CaBP1 m1CS2, indicating that, even under these conditions, the first trx box contributes the major refolding activity (Figure 5a). Interestingly, the state of the first box seems also to play a predominant role in the stability of CaBP1: whereas recombinant CaBP1 mutants containing only a mutation in the second box could be purified in equivalent amounts compared with the WT protein, the purification of recombinant CaBP1 m1, CaBP1 m1CS2 or CaBP1 m1,2 gave much lower yields, although the extent of expression by *E. coli* was equivalent to that of the other mutants (results not shown). This results most likely from a lower stability of CaBP1 species with mutations in the first trx box.

In contrast with CaBP1, destruction of either one of the three trx boxes of CaBP2 reduced the activity of the protein to a similar extent (Figure 4). When two boxes were inactivated the refolding activity was further reduced. Thus it is likely that, in CaBP2, the three boxes contribute to a similar extent to the oxidative refolding activity of CaBP2.

RNase AIII renaturation by PDI is inhibited in the presence of WT and mutated forms of CaBP1

In contrast with PDI, why was neither CaBP1 nor CaBP2 able to re-activate denatured reduced RNase AIII completely? We had previously observed [26] an over-additive acceleration of RNase AIII renaturation in the presence of PDI + CaBP1 or PDI + CaBP2. But this effect was only visible during the first 7–8 min after initiating the reaction. In these experiments the concentrations of PDI and CaBP1 or CaBP2 were 0.7 μM. We have re-examined this effect using WT CaBP1 and various CaBP1 mutants over 40 min, but using concentrations of 10 μM. The concentration of PDI used was always 5 μM.

Whereas with PDI alone a full renaturation of RNase AIII occurred within 20–30 min, the maximum renaturation obtained in the presence of PDI + WT CaBP1 was only about 60–70 % of total initial RNase AIII activity (Figure 6a). When PDI was combined with CaBP1 m2, only about 50 % of the initial RNase AIII activity was reached (Figure 6b). In both cases the time required to reach maximum re-activation (20–30 min) was the same as with PDI alone.

The extent of a decrease in maximum re-activation did not correlate with the renaturing activities of the various CaBP1 mutants: in the presence of both CaBP1 CS and PDI the decrease in yield of reactivated RNase was similar to that obtained with CaBP1 m2 and PDI (Figure 6c), although CaBP1 CS was only 50 % as active as WT CaBP1 or CaBP1 m2 when tested alone in the RNase AIII-renaturation assay.

Refolding of scRNase AIII in the presence of PDI, CaBP1, CaBP2 or their mutants

Since the function of PDIs is not only to enhance disulphide-bridge formation but also to isomerase potentially 'wrong' disulphide bridges, the ability of CaBP1 and CaBP2 to catalyse the formation of active RNase AIII from scRNase AIII was also analysed (Figure 7). Whereas PDI catalysed a 100 % re-activation of reduced, denatured RNase AIII, it led only to a partial renaturation (about 60 %) of scPDI (Figure 7a), indicating that, during the preparation of scRNase AIII, a considerable percentage of the protein had become irreversibly inactivated, most likely due to aggregation. Nevertheless, as observed for the folding of denatured reduced RNase, PDI displayed a much stronger activity than CaBP1 or CaBP2. While WT CaBP2 still had a significant refolding activity (about 30 % refolding after 40 min), that of WT CaBP1 was very low (Figure 7a). But even for this low activity a dependency on protein concentration was observed (results not shown). Surprisingly, even in the scRNase re-activation assay, the difference between the two trx boxes of CaBP1 became evident: while WT CaBP1 had only a very low, and CaBP1 m1 almost no, refolding activity, the CaBP1 m2 mutant exhibited a significant refolding activity when tested at concentrations between 5 and 20 μM (Figure 7b).

Renaturation of scRNase AIII by CaBP2 m1,2,3 could not be detected. When one or two trx boxes of CaBP2 were left intact, a small unscrambling activity was measured, but the differences between the various mutants were too small to justify conclusions as to different activities of the single boxes (results not shown).

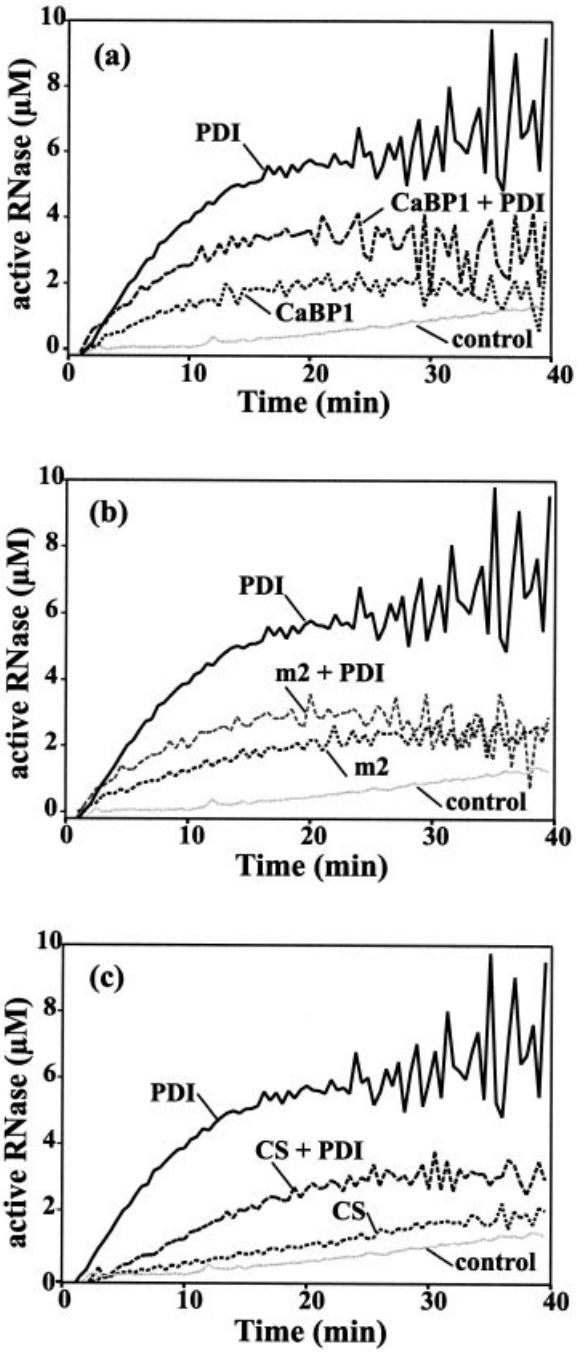

Figure 6 Efficiency of refolding of denatured reduced RNase AIII catalysed in concert by PDI and mutants of CaBP1

See the legend to Figure 1 for abbreviations. In the presence of WT or mutant CaBP1 (10 μM each), the yield of active RNase catalysed by PDI is significantly reduced: (**a**) 5 μM PDI and 10 μM CaBP1 result in a decrease of the yield to $\approx$ 65% that of the PDI-catalysed reaction without CaBP1; (**b**) substituting CaBP1 wt for m2, the yield of active RNase in the presence of PDI decreases further from 65 to 50%; m2 has an activity comparable with that of CaBP1 WT; (**c**) although the CS mutant alone has only 50% of the refolding activity of WT CaBP1, its effect on the reactivation of RNase in the presence of PDI is similar to that of the WT. Conditions for the negative control were as described above.

Complementation of PDI disruption by rat CaBP1 and CaBP2 genes

Oxidative folding of denatured RNase AIII can be carried out not only by PDI, but also by CaBP1 and CaBP2, but this does not necessarily mean that these rat proteins can replace the vital functions of PDI in cells. Since the yeast PDI gene is essential and since it had been shown that the lethality of a PDI disruption can be complemented by members of the PDI family [12,13], we asked whether expression of CaBP1 could also replace PDI

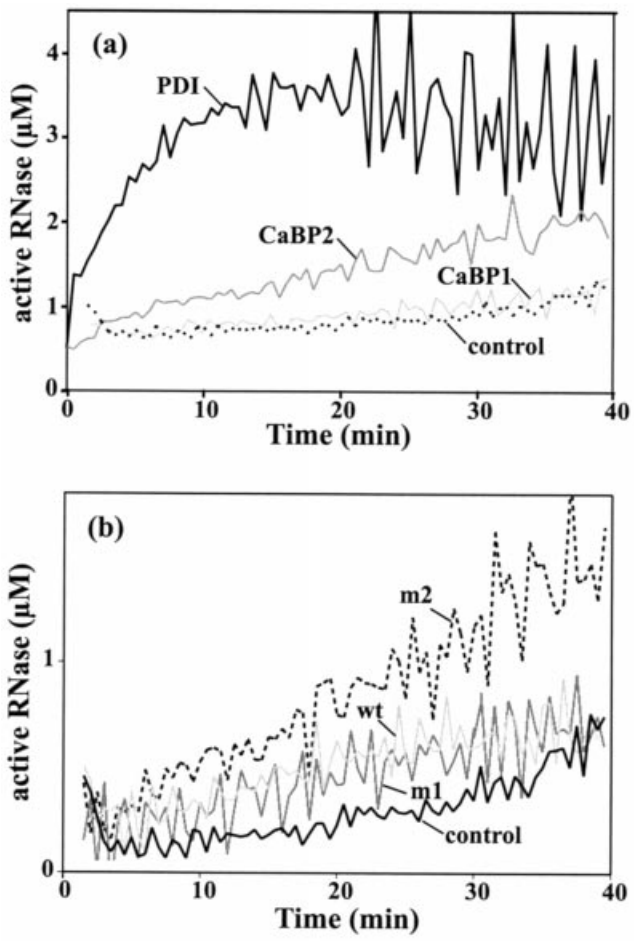

Figure 7 Effects of CaBP1, CaBP2 and PDI on the renaturation of scRNase AIII

(**a**) The maximum yield of renatured RNase in the presence of PDI was 60%, while CaBP2 generated 30%. CaBP1, however, had a very low, but detectable, activity (see below). PDI, CaBP1 and CaBP2 were employed at a final concentration of 5 μM each. (**b**) CaBP1 m2 displays a higher RNase unscrambling activity than WT, whereas the activity of m1 is even lower than that of WT. Here, all CaBP1 proteins were used at 10 μM final concentrations.

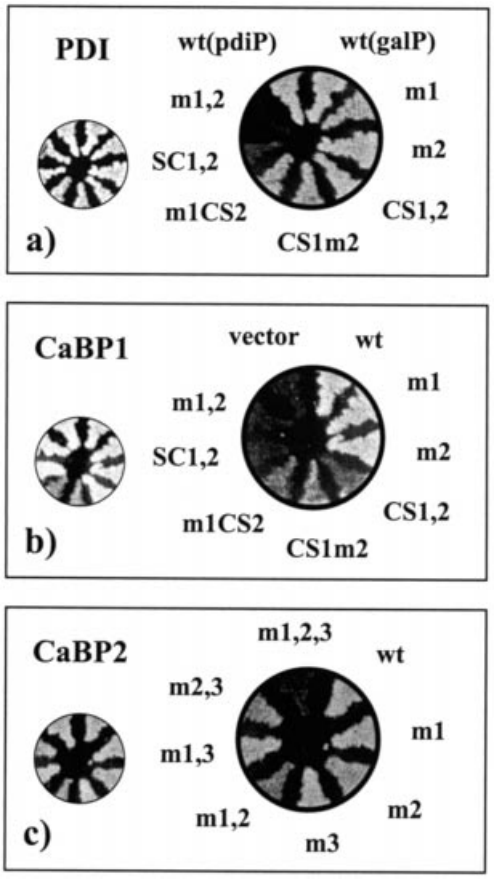

Figure 8 Complementation of the lethal PDI deficiency of *S. cerevisiae* by CaBP1 and CaBP2: comparison with PDI

See the legend to Figure 1 for abbreviations. Plates on the left show growth on selective media (without uracil and histidine). Plates on the right show growth on 5-FOA-containing media.

functionally in yeast, and, if so, how CaBP1 and CaBP2 with different mutations in their trx boxes might be able to complement.

We therefore constructed the tester strain BK203-15B, in which the PDI gene was disrupted by the *LEU2* gene; the WT PDI gene was present on a centromeric plasmid carrying the *URA3* gene as a selectable marker. In order to test the rat genes, they were placed under the control of the yeast gal1 promoter [43] in the yeast 2μ plasmid pDS [12]. Also, the rat retrieval signal sequences Lys-Glu-Glu-Leu and Lys-Asp-Glu-Leu respectively, were replaced by the yeast ER retrieval signal His-Asp-Glu-Leu. As a positive control a plasmid bearing the yeast PDI gene under control of the gal promoter was used. The tester strain BK203-15B was then transformed by the plasmids to be tested and individual transformant colonies were grown on histidine-free SC medium in the presence of 2% galactose. After transfer to medium containing 5-FOA, growth indicated that the cells were able to survive in the absence of the PDI. We found that not only the *CaBP2* gene, but also the *CaBP1* gene, was able to complement the PDI disruption (Figure 5).

Complementation was not seen in the absence of galactose, where expression from a 2μ plasmid is greatly reduced, but still strong enough to allow growth of strains carrying the PDI gene controlled by the gal promoter. It has been shown for PDI that inactivation of the trx boxes leads to cell death [9] and that the activity crucial for cell survival seems to be the isomerase activity of PDI, which is linked to the presence of the first cysteine residue in the trx boxes [11]. In light of the results obtained from the *in vitro* folding of scRNase AIII, where the CaBP1 WT protein displays only a very weak isomerase activity and where no detectable activity was seen with the CaBP1 m1CS2 mutant (results not shown), the question arose whether the ability of CaBP1 and CaBP2 to complement the PDI disruption also depended on the integrity of their trx boxes. Thus the different CaBP1 and CaBP2 mutants were subjected to the complementation assay. As a control, corresponding mutations were also introduced into the PDI gene and tested (Figure 8a). Similarly to PDI, the ability of CaBP1 or CaBP2 to replace PDI functionally was completely abolished when all cysteine residues in the trx boxes were mutated to serine residues (Figures 8b–8c), but complementation was restored with mutants in which one (CaBP1 and CaBP2) or two (CaBP2) of the different trx boxes were left intact (Figures 8b–8c).

Analysing the mutants of PDI and CaBP1 in which either the first or the second cysteine residue of the trx boxes was replaced

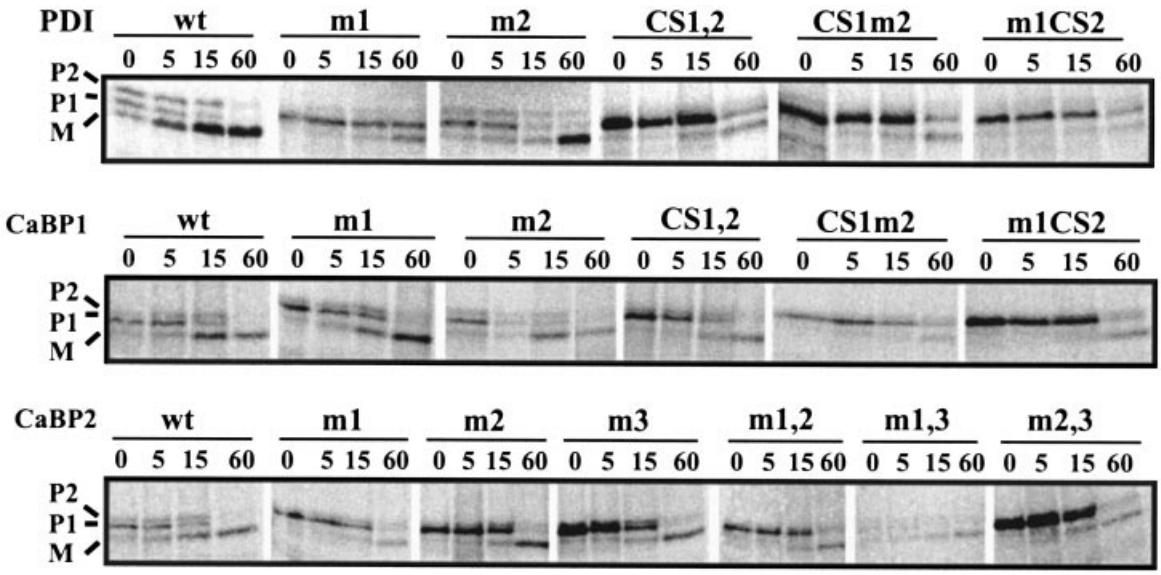

Figure 9 Processing of carboxypeptidase Y by PDI, CaBP1 and CaBP2

Autoradiograph of SDS/PAGE-separated product from a pulse–chase experiment. Samples were taken after 0, 5, 15 and 60 min as indicated. P1, P2 and M refer to glycosylated precursor species (67 kDa ER form and 69 kDa Golgi form) and the mature, 62 kDa vacuolar form of CPY respectively. See the legend to Figure 1 for abbreviations of proteins. In the presence of WT PDI (top panel), CaBP1 (middle panel) and CaBP2 (lower panel), CPY is processed normally with the M form dominating after 15 min. Disruption of the second trx-box of CaBP1 (m2) had less of an effect on maturation of CPY than disruption of the first box (m1). For CaBP2, the effect on CPY processing was the same for all single trx-box mutations (m1, m2 or m3), resulting in more severe retardation of processing than CaBP1m1 or m2. Disruption of a second box in CaBP2 led to only slightly slower processing of CPY compared with m1, m2 or m3.

by serine (PDI CS, PDI CS1m2, PDI m1CS2, PDI SC, CaBP1 CS, CaBP1 CS1m2, CaBP1 m1CS2 and CaBP1 SC) demonstrated that, not only for PDI, but also for CaBP1, the N-terminal cysteine residues of the boxes play the crucial role for the survival of the yeast cells (Figures 8b–8c). However, growth of cells expressing mutants in which only one N-terminal cysteine residue was left intact (CaBP1 CS1m2 or CaBP1 m1CS2) was severely retarded (Figures 8b–8c). These results indicate that the isomerase activities of CaBP1 and CaBP2 play an essential role, not only in the *in vitro* refolding assay, but also in the complementation of the growth defect in yeast cells with a disrupted PDI gene.

CPY processing in yeast expressing genes coding for PDI, CaBP1, CaBP2 or mutants of these proteins

For an analysis of folding processes catalysed by CaBP1 and CaBP2 and the respective mutants *in vivo*, we chose as a model the processing of yeast CPY, which can be monitored by separating the ER, Golgi and vacuolar forms of the enzyme by SDS/PAGE as proteins of apparent molecular masses of 67, 69 and 62 kDa respectively. We included also PDI and its mutants in the present study, since so far a detailed analysis of *in vivo* processing of CPY catalysed by trx-box mutants of PDI had been lacking.

Cells containing either WT or mutant PDI, CaBP1 or CaBP2 were subjected to pulse–chase experiments. Only those mutants that had complemented the PDI deletion in the growth assay were analysed.

In cells expressing WT PDI, more than 50% of CPY was found as the mature form after 15 min, and about 90% after 60 min (Figure 9a). Maturation of CPY was only slightly lower with mutant PDI m2, where the first trx box was left intact and the second contained two serine residues instead of cysteine residues (Figure 9a). On the other hand, cells expressing mutant PDI m1 (first box mutated) displayed a delayed CPY processing

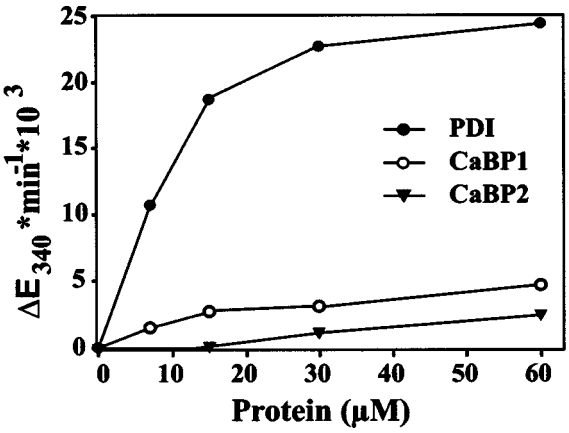

Figure 10 Effect of members of the PDI family on the non-oxidative refolding of GAPDH

GAPDH, denatured in 4.5 M guanidinium chloride as described in [16], was diluted to 2.8 μM in assay buffer containing 0–30 μM of PDI, CaBP2 or CaBP1. Re-activation of the enzymic activity of GAPDH (measured at 340 nm as the reduction of NAD^+ to NADH; $\Delta E_{340} = \Delta A_{340}$) was taken as a measure of the extent of renaturation. As a negative control, denatured GAPDH was diluted as described above, but into a solution lacking protein. The measured activity of renatured GAPDH is plotted against the concentration of the refolding enzyme. The activity of PDI-catalysed renatured GAPDH was about 20% that of the positive control (untreated native GAPDH; results not shown). Both CaBP1 and CaBP2-catalysed reactions were in the same range as the negative control (denatured GAPDH without refolding enzyme; results not shown).

(only $\approx 30\%$ after 15 min and $\approx 50\%$ after 1 h) (Figure 9a). The rate of processing was even further reduced in mutant PDI m1CS2, where only the first cysteine residue in the second trx box

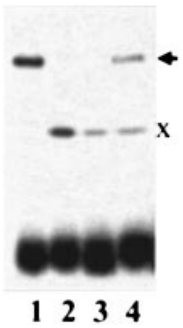

Figure 11 Radiolabelled Δ-somatostatin interacts with PDI but not with CaBP2

[^{125}I]Bolton–Hunter-reagent-labelled Δ-somatostatin was incubated with *E. coli* lysates, expressing either PDI or CaBP2, and chemical cross-linker. An *E. coli* lysate that did not express any PDIs served as a control. An arrow indicates relevant cross-linking products on the autoradiograph. X, intrinsic cross-linking product to an unidentified, endogenous *E. coli* protein. PDI (lane 1) clearly binds the labelled peptide, whereas CaBP2 (lane 3), as the control (lane 2) does not. Binding of the peptide is not restricted to PDI alone, however; another PDI protein, PDIp, also binds labelled Δ-somatostatin (lane 4).

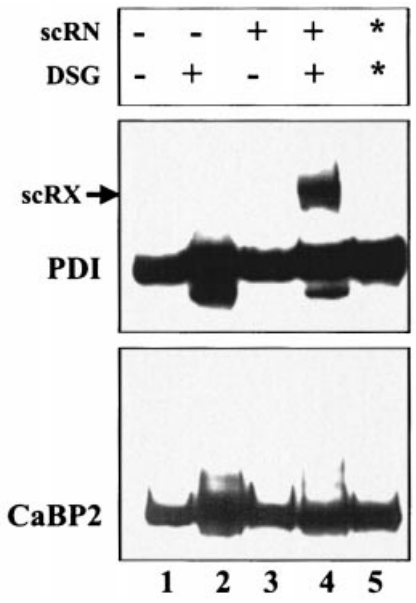

Figure 12 ScRNase A interacts with PDI, but not with CaBP2

E. coli lysates expressing PDI and CaBP2 respectively were incubated in the presence or absence of scRNase A ('scRN') (3 μM) with or without the chemical cross-linker DSG ('DSG') as indicated. Endogenous PDI ('PDI') and CaBP2 ('CaBP2') and the cross-linking product ('scRX') were detected after Western blotting and staining with specific antibodies raised against PDI and CaBP2 respectively. Clearly a cross-linked complex can be observed for PDI–scRN but not for CaBP2–scRN (lane 4).

was left (≈ 20 % after 15 min, ≈ 40 % or less after 1 h) (Figure 9a).

Processing of CPY in cells expressing WT CaBP1 or WT CaBP2 was comparable with that seen in cells expressing WT PDI with about 50 % mature CPY after 15 min and about 90 % after 1 h, indicating that the function of PDI in CPY folding can be taken over by CaBP1 or CaBP2 (Figures 9b–9c). The analysis of CaBP1 with mutated trx boxes revealed that processing of CPY was faster with mutants where the second box had been mutated (compare 15 min samples of CaBP1 m1 and CaBP1 m2). The processing of CPY was extremely reduced, but was still detectable in mutants containing only one 'half trx box' (CaBP1 CS1m2 and CaBP1 m1CS2) (Figure 9b). This correlates well with the low growth rate of cells with a disrupted PDI gene expressing these mutated proteins.

Analysis of CaBP2 mutants shows that inactivation of even a single trx box retarded CPY processing. But the degree of retardation was similar for CaBP2 m1, CaBP2 m2 and CaBP2 m3 (Figure 9c). This parallels the results obtained for the refolding of RNase AIII. CaBP2 mutants with only one intact trx box showed only a slightly higher degree of retardation of CPY maturation than the mutants with two intact boxes (Figure 9c). Again, the three mutants with only one intact trx box did not exhibit detectable differences (Figure 9c). Both findings agree with the results obtained from the refolding of reduced denatured RNase AIII.

Yeast cells bearing derivatives of CaBP1, CaBP2 or PDI without functional trx boxes could not be tested with respect to CPY folding as these cells did not grow. However, the fact that mutants of PDI or CaBP1 with only one 'half-box' left had only a very low CPY-folding activity, makes it likely that proteins of the PDI family with a complete lack of functional trx boxes will no longer promote folding of CPY.

Comparison of trx-box-independent activities of PDI, CaBP2 and CaBP1: ability to bind malfolded peptide or protein substrates with or without disulphide bonds

Although our results strongly support the view that the essential function of PDI is associated with trx boxes, trx-box-independent chaperone properties of PDI have been demonstrated *in vitro* [16,17] and *in vivo* [18–20]. Assuming that chaperone activities might significantly assist these essential functions, one might expect that not only PDI, but also CaBP2 or CaBP1, could share these chaperone activities. GAPDH does not contain disulphide bridges, and the renaturation therefore cannot involve an isomerase function. While PDI clearly exhibited a renaturing effect, neither CaBP2 nor CaBP1 exhibited any activity in this respect (Figure 10). Furthermore, neither protein had any effect on the PDI-catalysed renaturation of GAPDH (results not shown).

PDI clearly binds peptides such as Δ-somatostatin, as seen by cross-linking with DSG (Figure 11). Whereas we found a similar peptide-binding activity with another PDI protein, PDIp, no interaction with CaBP2 could be seen. In addition, other cross-linking experiments clearly showed (Figure 12) that PDI, but not CaBP2, interacts with a denatured, but oxidized, substrate (scRNase AIII).

DISCUSSION

We show here that not only CaBP2, but also CaBP1, can complement growth in *S. cerevisiae* with a disrupted PDI gene. The question was whether these effects of CaBP2 and CaBP1 require the intact trx boxes or whether other or additional properties, e.g. chaperone functions, contribute to their complementing activities.

In agreement with our previous results [26], WT CaBP1 and WT CaBP2 catalysed the refolding of reduced, denatured RNase AIII. However, while PDI catalysed a full re-activation of RNase AIII, re-activation in the presence of CaBP2 or CaBP1 did not exceed 70 %, even after prolonged incubation.

In the presence of PDI + CaBP1 the final yield of active RNase AIII was significantly lower than in the presence of PDI alone.

A similar effect was seen when PDI was combined with mutated CaBP1 which had either very low, or no, RNase AIII-renaturing activity.

After prolonged incubation, the re-activation of RNase in the presence of both PDI and CaBP1 levels off and does not increase further, remaining significantly lower than the levels of re-activation in the presence of PDI alone. This argues against a simple competitive inhibition of PDI, since the levels of RNase activity would then be expected to continue to rise slowly. Stable binding to PDI is an unlikely explanation – we were unable to detect significant PDI–CaBP1 complexes in cross-linking assays (results not shown) – and any other form of binding would necessarily allow a pool of free PDI to exist that would then lead to a gradual increase in the levels of re-activated RNase rather than the observed levelling off.

CaBP1 or CaBP2 in which all cysteine residues of the trx boxes had been converted into serine residues were completely inactive with respect to renaturation of denatured reduced RNase AIII as well as with respect to complementation of the growth defect. One intact trx box in either protein was sufficient to allow growth and to promote RNase AIII re-activation. In accordance with data obtained for PDI [42], mutants of CaBP1 in which only the N-terminal cysteine residues of the two trx boxes were preserved still exhibited a significant activity in supporting RNase AIII re-activation as well as growth, indicating the rate-limiting role of the isomerization reaction under both conditions.

Although neither the growth assay nor the CPY maturation assay could demonstrate possible differences between the individual trx boxes of either CaBP1 or CaBP2, a compilation of the results of the *in vitro* assays provided some interesting information. To understand these results, one should keep in mind that the two 'RNase' assays – the refolding of denatured, reduced RNase AIII and the re-activation of scRNase AIII – involve many common steps. Thus oxidation of denatured, reduced RNase produces oxidized RNase that undergoes isomerization reactions similar to those that occur in the reshuffling of disulphide bonds in scRNase.

Imagine the scheme shown below:

$$U_{red} \rightarrow\rightarrow U_{ox} \leftrightarrow\leftrightarrow U_{ox(2)} \leftrightarrow\leftrightarrow U_{ox(n)} \rightarrow\rightarrow N_{ox}$$

U_{red} represents the reduced, denatured RNase. U_{ox} is the initial oxidized RNase produced by the redox active enzyme. $U_{ox(2)}$ and $U_{ox(n)}$ represent species of RNase with variable numbers of isomerized disulphide bonds. N_{ox} represents the native, oxidized RNase AIII. Arrows represent enzyme-catalysed steps, which are either reversible ($\leftrightarrow$) or irreversible ($\rightarrow$).

PDI catalayses this reaction to completion. CaBP1, however, produces only 50 % N_{ox}. But, in the presence of PDI, the yield of N_{ox} is $\approx$ 70 %. The remaining 30 % cannot be converted into an active RNase AIII species and is irretrievable. This fraction might be an intermediate in the PDI-catalysed reaction, but it cannot bind PDI after being produced by CaBP1, perhaps by its swift conversion into inaccessible aggregates. Thus for CaBP1 we would have:

$$\begin{array}{l} U_{red} \rightarrow\rightarrow U_{ox} \leftrightarrow\leftrightarrow U_{ox(2)} \leftrightarrow\leftrightarrow U_{ox(n)} \rightarrow\rightarrow N_{ox} \\ \qquad\qquad\qquad\quad \downarrow \\ \qquad\qquad\qquad\quad U_{ox(z)} \end{array}$$

where $U_{ox(z)}$ (at $\approx$ 30 % in the presence of PDI) cannot serve as substrate for either CaBP1 or PDI.

The CaBP1 mutant m2 (in which both cysteine residues of the second trx box are mutated to serine residues) has the same overall activity as WT CaBP1. That is, N_{ox} produced is identical, even though m2 has one completely inactivated trx box. However, in the presence of PDI, the yield is lower than with PDI and WT CaBP1 [i.e. $U_{ox(z)} > 30$ %]. Thus the species $U_{ox(z)}$ is produced in proportionally greater amounts by the m2 mutant than by WT CaBP1. The m1 mutant, by contrast, catalyses refolding of only 25 % active RNase.

Now CaBP1 CS is a mutant that has both Cys-Gly-His-Cys boxes mutated to Cys-Gly-His-Ser. In the oxidative folding of RNase, it is nevertheless 50 % as active as WT CaBP1 (i.e. 25 % N_{ox}). Furthermore, in the presence of PDI, the yield of RNase is as great as with WT and PDI [70 % N_{ox}, 30 % $U_{ox(z)}$]. Here the proportion of unusable $U_{ox(z)}$ seems to remain unchanged ($\approx$ 30 %). Thus the more N-terminal Cys residue of either or both of the two trx boxes is sufficient for the formation of all of the $U_{ox(z)}$ formed by WT CaBP1.

CaBP1 CS1m2 has a low activity in the refolding of reduced, denatured RNase, yet CS2m1 has no such activity. This indicates that the CS1m2 protein can isomerize but also oxidize, whereas CS2m1 can only isomerize. Thus, in CaBP1 CS, the first trx box CGHS carries out most or all of the oxidation under these conditions and both boxes carry out isomerization. Here, oxidation catalysed via the single active-site cysteine residue is likely to proceed via GSSG present in the buffer, although some air oxidation of the substrate could occur as well. In other words, in CaBP1, the N-terminal trx box is more oxidizing than the C-terminal trx box.

The above arguments are all in keeping with the observation that scRNase is not fully re-activatable by PDI (60 % N_{ox}). Some $U_{ox(z)}$ species are likely to have formed at random during the preparation of RNase, and are not retrievable. [In addition, PDI may promote formation of $U_{ox(z)}$ by isomerization of other unusual intermediates.] Thus, in the refolding of reduced denatured RNase, PDI prevents losses by preventing $U_{ox(z)}$ from ever forming – most likely by a direct interaction with the substrate via non-trx-box peptide-binding sites, i.e., a chaperone function.

CaBP2, and especially CaBP1, produce a lot more of the abortive species [$U_{ox(z)}$], in keeping with our previous conclusions on the refolding of reduced, denatured RNase. Assuming similar activity as in the refolding of reduced RNase (i.e. 50 % of PDI activity, due to formation of abortive species), a crude estimate of the yield of active RNase catalysed by CaBP1 WT and the m2 mutant (at 2.8 μM each) in the unscrambling assay would be in the range $\approx$ 25–30 %. However, no significant unscrambling activity of any of the CaBP1 proteins could be measured at this concentration. Even after increasing the concentrations of CaBP1 proteins by 3.5-fold to 10 μM, only CaBP1 m2 ($\approx$ 25 %), but not WT CaBP1 ($\approx$ 10 %), approaches the estimated value after 40 min. The m1 mutant (estimated 10–15 % activity) has an even lower, barely detectable, activity. Thus, not only is the pool of scRNase that can serve as a substrate for CaBP1 greatly reduced, but the yield of activated RNase catalysed by CaBP1 m2 now exceeds that catalysed by CaBP1 WT.

Remaining questions now phrase themselves: (1) why, in the reduced RNase assay, is the activity of the second trx box of CaBP1 (as measured with m1) similar to CaBP1 CS (25 % N_{ox}), yet, in the WT protein, not be additive with the activity of the first trx box (the m2 mutant)? (2) Similarly, why, in the scRNase assay, does the integrity of the second trx box result in a much lower than expected activity of WT when the second trx box alone possesses literally no activity? Here, we do not have additional data for PDI + CaBP1 m1, but one clue presents itself for speculation by the inquisitive investigator: the ratio of WT activity to the activity of CaBP1 m2 is lower in the unscrambling assay than in the denatured, reduced RNase folding assay. In

other words, CaBP1 may be far from inactive in the unscrambling assay. Rather, mainly the second trx box may (1) actively catalyse the formation of an inaccessible species of RNase either by isomerizing free scRNase or that produced by either trx box to $U_{ox(z)}$ or (2) form abortive CaBP1–RNase intramolecular disulphide-bonded complexes to such an extent that it reduces the activity of WT CaBP1, especially in the 'scrambled' assay.

The CaBP1–RNase kinetically trapped species would involve a (highly) oxidized RNase molecule disulphide-bonded to the second trx box of CaBP1. Predominant 'isomerase only' activity of the second trx box and the formation of heterotypic disulphide-linked species would also account for (1) the relatively low effect of the second, intact trx box on WT activity in the reduced RNase assay in contrast with the greater, more unproductive effect in the scRNase assay, caused by an immediate excess of substrate (highly oxidized, i.e. scRNase) in the latter case, (2) the lack of productive activity of m1 in the 'scrambled' assay compared with the low activity of m1 in the 'reduced' assay.

In point (1) above, the presence of substrate at the second trx box may lower activity of the first box by (spatially) preventing access of substrate to the first trx box or by thereby reducing the free pool of substrate RNase significantly (the K_m for the PDI-catalysed re-activation of reduced, denatured RNase is $\approx 6.6\ \mu$M [42]; a value for scRNase in the presence of GSH is of the order of ≈ 0.5 mM [31,45]).

This suggestion is in line with recent findings that escape from heterotypic disulphides between PDI and scRNase catalysed by a second active-site cysteine residue is the dominant mechanism for PDI-dependent isomerization of RNase [46]. Although m1 has two cysteine residues in its second box, it behaves as though only one cysteine residue were present – namely it lacks oxidative capacity. A highly oxidized substrate RNase is very slow in rearranging and offers little hope of rescue to an m1 mutant thus covalently bound. In the presence of PDI, however, the bound RNase could be rescued.

The apparent paradox of this unproductive activity in the WT protein co-existing with a productive activity when the m1 mutant is used alone in the reduced RNase assay, and the obvious predominance of the unproductive activity in the unscrambling assay could thus have its causal agent in the *modus operandi* of a unifying catalytic mechanism. The functional non-equivalence of the trx boxes in CaBP1 could be due to differing redox potentials of the two active sites, a point illustratively supported throughout the present paper.

The fact that no cross-linked products could be observed between malfolded RNase and CaBP2 suggests that the lower yield is not due to the formation of abortive CaBP2–RNase complexes, but rather to the formation of abortive RNase species lacking CaBP2. CaBP1 could not be used in the cross-linking assays, as the recombinant protein could not be stably expressed from the pET12 construct. Thus we cannot yet verify whether the low yield of active RNase catalysed by CaBP1 is due to the production of a kinetically trapped RNase species that involves heterotypic disulphide-bonding or aggregate formation between CaBP1 and RNase, or, as might be extrapolated from the absence of CaBP2/RNase cross-linked products, whether these trapped species are homotypic in character. Although all trx boxes in CaBP2 seem equivalent, and all have the capacity to oxidize substrate as well as isomerize, a possible preferential formation of a trapped, cross-linked species would be unique to the second trx box of CaBP1.

PDI, but not CaBP2 or CaBP1, exerted a chaperone function in the refolding of denatured GAPDH. Indeed, other experiments showed that human ERp28, a PDI-family member that naturally lacks reactive cysteines [2,44], as CaBP1 and CaBP2, had no effect on the re-activation of GAPDH (results not shown), although it has been shown to interact with a malfolded substrate *in vivo* [44]. Thus PDI itself is the only protein able to catalyse the re-activation of GAPDH, and the chaperone activity associated with the C-terminal end of PDI [16] may be unique to PDI alone. Similarly, peptide-binding activity, which in PDI is attributed mainly to the b′ domain [5], seems to be lacking in CaBP2. As the b′ domain of PDI is essential, though not sufficient, for the binding of larger polypeptides to PDI, and as RNase binds to a PDI fragment containing the b′–a′ domains but not to fragments lacking either of these domains [5], the inability of CaBP2 to bind denatured scRNase, as seen by cross-linking, indicates that CaBP2 lacks a corresponding 'major peptide-binding site'.

We can now attempt to speculate on the nature of the abortive RNase species, $U_{ox(z)}$. This species, irretrievable even for PDI once produced, is formed preferentially via the high oxidative power of CaBP1 trx box 1 and is unlikely to be composed of disulphide-linked RNase–CaBP1 complexes (trx box 1 can effectively escape from disulphide interactions as does PDI and CaBP2). Thus $U_{ox(z)}$ could be an oxidized, malfolded RNase species formed both by CaBP1 and CaBP2 due to the lack of peptide-binding sites that in PDI prevent $U_{ox(z)}$ from ever forming and/or from undergoing irreversible aggregation.

In the CPY-maturation assay, both CaBP1 and CaBP2 had a similar activity as transfected PDI in the presence of galactose. Removal of a single trx box in either CaBP1 or CaBP2 was already sufficient to lead to retardation of CPY maturation. Again, the retardation with CaBP1 m2 was less than with CaBP1 m1 (Figure 9).

In contrast, destruction of one trx box in CaBP2 led to similar extents of retardation of CPY maturation irrespective of the position of the box, as had already been observed in the RNase AIII-re-activation assay. In CaBP2, therefore, the trx boxes seem functionally equivalent.

While the activities of CaBP1 and CaBP2 in the RNase AIII re-activation assay were clearly lower than that of PDI, in the presence of galactose neither the growth assay nor the CPY-maturation assay showed significant differences between the activities of the three WT proteins. The reason for this discrepancy could be that CPY folding intermediates, in contrast with RNase folding intermediates, are not susceptible to inactivation during CaBP1- or CaBP2-catalysed folding or that inactivation is suppresed by some unknown factor within the ER.

In conclusion, the lower efficiencies of CaBP1 and CaBP2 in productive refolding of a reduced substrate (RNase AIII) seems likely to be due to their inability to (sufficiently) interact with the substrate via peptide-binding sites, thereby directly or indirectly promoting the formation of abortive RNase species. These data do not preclude the existence of peptide-binding sites in either CaBP1 or CaBP2, but, taken together with the lack of refolding of denatured GAPDH and the inability to bind scRNase or Δ-somatostatin, this does strongly suggest that (1) both CaBP1 and CaBP2 differ importantly from PDI in lacking a general peptide-binding site, (2) such a site is crucial in increasing productive oxidative folding, and (3) in the absence of such a site, CaBP1 and CaBP2 promote folding of RNase via different kinetic pathways.

Although our data suggest the lack of a general peptide-binding site in either CaBP1 or CaBP2, the presence of inactivated b-type domains in both of these proteins could be an indication of the existence of peptide-binding sites for a more selective range of substrates. For these particular substrates, a lower rate of redox folding, determined, perhaps, by specific interaction with a preferred trx box of CaBP1, may be advantageous.

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REFERENCES

1 Goldberger, R. F., Epstein, C. J. and Anfinsen, C. B. (1963) Acceleraion of reactivation of reduced bovine pancreatic ribonuclease by a microsomal system from rat liver. J. Biol. Chem. **238**, 628–635

2 Ferrari, D. M. and Söling, H. D. (1999) The protein disulphide isomerase family: unravelling a string of folds. Biochem. J. **339**, 1–10

3 Noiva, R., Freedman, R. B. and Lennarz, W. J. (1993) Peptide binding to protein disulfide-isomerase occurs at a site distinct from the active-sites. J. Biol. Chem. **268**, 19210–19217

4 Darby, N. J., Penka, E. and Vincentelli, R. (1998) The multi-domain structure of protein disulfide isomerase is essential for high catalytic activity. J. Mol. Biol. **276**, 239–247

5 Klappa, P., Ruddock, L. W., Darby, N. J. and Freedman, R. B. (1998) The b′ domain provides the principle peptide-binding site of protein disulfide isomerase but all domains contribute to binding of misfolded proteins. EMBO J. **17**, 927–935

6 Koivunen, P., Pirneskoski, A., Karvonen, P., Ljung, J., Helaakoski, T., Notbohm, H. and Kivirikko, K. I. (1999) The acidic C-terminal domain of protein disulfide isomerase is not critical for the enzyme subunit function or for the chaperone or disulfide isomerase activities of the polypeptide. EMBO J. **18**, 65–74

7 Dai, Y. and Wang, C. (1997) A mutant truncated protein disulfide isomerase with no chaperone activity. J. Biol. Chem. **272**, 27572–27576

8 Wunderlich, M., Otto, A., Maskos, K., Mucke, M., Seckler, R. and Glockshuber, R. (1995) Efficient catalysis of disulfide formation during protein folding with a single active-site cysteine. J. Mol. Biol. **247**, 28–33

9 Laboissière, M. C. A., Sturley, S. L. and Raines, R. T. (1995) The essential function of protein disulfide isomerase is to unscramble non-native disulfide bonds. J. Biol. Chem. **270**, 28006–28009

10 Darby, N. J. and Creighton, T. E. (1995) Functional properties of the individual thioredoxin-like domains of protein disulfide isomerase. Biochemistry **34**, 11725–11735

11 Walker, K. W., Lyles, M. M. and Gilbert, H. F. (1996) Catalysis of oxidative protein folding by mutants of protein disulfide isomerase with a single active-site cysteine. Biochemistry **35**, 1972–1980

12 Günther, R., Srinivasan, M., Haugejorden, S., Green, M., Ehrbrecht, I. M. and Küntzel, H. (1993) Functional replacement of the *Saccharomyces cerevisiae* Trg1/Pdi1 protein by members of the mammalian protein disulfide isomerase family. J. Biol. Chem. **268**, 7728–7732

13 Tachikawa, H., Takeuchi, Y., Funahashi, W., Miura, T., Gao, X. D., Fujimoto, D., Mizunaga, T. and Onodera, K. (1995) Isolation and characterization of a yeast gene, *MPD1*, the overexpression of which suppresses inviability caused by protein disulfide isomerase depletion. FEBS Lett. **369**, 212–216

14 Chivers, P. T., Laboissière, M. C. A. and Raines, R. T. (1996) The CXXC motif: imperatives for the formation of native disulfide bonds in the cell. EMBO J. **15**, 2659–2667

15 Lundström, J., Krause, G. and Holmgren, A. (1992) A Pro to His mutation in active site of thioredoxin increases its disulfide-isomerase activity 10-fold. New refolding systems for reduced or randomly oxidized ribonuclease. J. Biol. Chem. **267**, 9047–9052

16 Cai, H., Wang, C. C. and Tsou, C. L. (1994) Chaperone-like activity of protein disulfide isomerase in the refolding of a protein with no disulfide bonds. J. Biol. Chem. **269**, 24550–24552

17 Song, J. L. and Wang, C. C. (1995) Chaperone-like activity of protein disulfide isomerase in the refolding of rhodanese. Eur. J. Biochem. **231**, 312–316

18 Vuori, K., Myllylä, R., Pihlajaniemi, T. and Kivirikko, K. (1992) Expression and site-directed mutagenesis of human protein disulfide isomerase in *Escherichia coli*. This multifunctional polypeptide has two independently acting catalytic sites for the isomerase activity. J. Biol. Chem. **267**, 7211–7214

19 Hayano, T., Hirose, M. and Kikuchi, M. (1996) Protein disulfide isomerase lacking its isomerase activity accelerates protein folding in the cell. FEBS Lett. **377**, 505–511

20 Wetterau, J. R., Combs, K. A., Spinner, S. N. and Joiner, B. J. (1990) Protein disulfide isomerase is a component of the microsomal triglyceride transfer protein complex. J. Biol. Chem. **265**, 9800–9807

21 Whiteley, E. M., Hsu, T. A. and Betenbaugh, M. J. (1997) Thioredoxin domain non-equivalence and anti-chaperone activity of protein disulfide isomerase mutants *in vivo*. J. Biol. Chem. **272**, 22556–22563

22 Westphal, V., Darby, N. J. and Winther, J. R. (1999) Functional properties of the two redox-active sites in yeast protein disulfide isomerase *in vitro* and *in vivo*. J. Mol. Biol. **286**, 1229–1239

23 Nguyen Van, P., Peter, F. and Söling, H. D. (1989) Four intracisternal calcium-binding glycoproteins from rat liver microsomes with high affinity for calcium. J. Biol. Chem. **264**, 17494–17501

24 Füllekrug, J., Sönnischen, B., Wünsch, U., Arseven, K., Nguyen Van, P., Söling, H. D. and Mieskes, G. (1994) CaBP1, a calcium binding protein of the thioredoxin family, is a resident KDEL protein of the ER and not of the intermediate compartment. J. Cell Sci. **107**, 2719–2727

25 Mazzarella, R. A., Srinivasan, M., Haugejorden, S. M. and Green, M. (1990) ERp72, an abundant luminal endoplasmic reticulum protein, contains three copies of the active site sequences of protein disulfide isomerase. J. Biol. Chem. **265**, 1094–1101

26 Rupp, K., Birnbach, U., Lundström, J., Nguyen Van, P. and Söling, H. D. (1994) Effects of CaBP2, the rat analog of ERp72, and of CaBP1 on the refolding of denatured reduced proteins. J. Biol. Chem. **269**, 2501–2507

27 Nguyen Van, P., Rupp, K., Lampen, A. and Söling, H. D. (1993) CaBP2 is a rat homolog of ERp72 with protein disulfide isomerase activity. Eur. J. Biochem. **213**, 789–795

28 Kramer, W., Kramer, B., Williamson, M. S. and Fogel, S. (1989) Cloning and nucleotide sequence of DNA mismatch repair gene PMS1 from *Saccharomyces cerevisiae*: homology of PMS1 to prokaryotic MutL and HexB. J. Bacteriol. **171**, 5339–5346

29 Kunkel, T. A. (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. U.S.A. **82**, 488–492

30 Desilva, M. G., Lu, J., Donadel, G., Modi, W. S., Xie, H., Notkins, A. L. and Lan, M. S. (1996) Characterisation and chromosomal localisation of a new protein disulfide isomerase, PDIp, highly expressed in human pancreas. DNA Cell Biol. **15**, 9–16

31 Lyles, M. and Gilbert, H. (1991) Catalysis of the oxidative folding of ribonuclease A by protein disulfide isomerase: dependence of the rate on the composition of the redox buffer. Biochemistry **30**, 613–619

32 Gill, S. C. and Von Hippel, P. H. (1989) Calculation of protein extinction coefficients from amino acid sequence data. Anal. Biochem. **182**, 319–326

33 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248–254

34 Boeke, J. D., Trueheart, J., Natsoulis, G. and Fink, G. R. (1987) 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. Methods Enzymol. **154**, 164–175

35 Rothblatt, J. and Schekman, R. (1989) A hitchhiker's guide to analysis of the secretory pathway in yeast. Methods Cell Biol. **32**, 3–36

36 Rowling, P. J., McLaughlin, S. H., Pollock, G. S. and Freedman, R. B. (1994) A single purification procedure for the major resident proteins of the ER lumen: endoplasmin, BiP, calreticulin and protein disulfide isomerase. Protein Expression Purif. **5**, 331–336

37 Fleischer, S. and Kervina, M. (1974) Subcellular fractionation of rat liver. Methods Enzymol. **31**, 6–41

38 Klappa, P., Mayinger, P., Pipkorn, R., Zimmermann, M. and Zimmermann, R. (1991) A microsomal protein is involved in ATP-dependent transport of presecretory proteins into mammalian microsomes. EMBO J. **10**, 2795–2803

39 Klappa, P., Zimmermann, M. and Zimmermann, R. (1994) The membrane proteins TRAMp and sec61 alpha p may be involved in post-translational transport of presecretory proteins into mammalian microsomes. FEBS Lett. **341**, 281–287

40 Klappa, P., Freedman, R. B. and Zimmermann, R. (1995) Protein disulphide isomerase and a lumenal cyclophilin-type peptidyl prolyl *cis–trans* isomerase are in transient contact with secretory proteins during late stages of translocation. Eur. J. Biochem. **232**, 755–764

41 Klappa, P., Hawkins, H. C. and Zimmermann, R. (1997) Interaction between protein disulfide isomerase and peptides. Eur. J. Biochem. **248**, 37–42

42 Lyles, M. M. and Gilbert, H. F. (1994) Mutations in the thioredoxin sites of protein disulfide isomerase reveal functional nonequivalence of the N- and C-terminal domains. J. Biol. Chem. **269**, 30946–30952

43 Johnston, M. and Davis, R. W. (1984) Sequences that regulate the divergent GAL1-GAL10 promoter in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **4**, 1440–1448

44 Ferrari, D. M., Nguyen Van, P., Kratzin, H. D. and Soling, H. D. (1998) ERp28, a human endoplasmic-reticulum-lumenal protein, is a member of the protein disulfide isomerase family but lacks a CXXC thioredoxin-box motif. Eur. J. Biochem. **255**, 570–579

45 Lambert, N. and Freedman, R. B. (1983) Structural properties of homogeneous protein disulphide-isomerase from bovine liver purified by a rapid high-yielding procedure. Biochem. J. **213**, 225–234

46 Walker, K. W. and Gilbert, H. F. (1997) Scanning and escape during protein-disulfide isomerase-assisted protein folding. J. Biol. Chem. **272**, 8845–8848

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