DNA-binding Activity of the ERp57 C-terminal Domain Is Related to a Redox-dependent Conformational Change*

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ERp57, a member of the protein-disulfide isomerase family, although mainly localized in the endoplasmic reticulum is here shown to have a nuclear distribution. We previously showed the DNA-binding properties of ERp57, its association with the internal nuclear matrix, and identified the C-terminal region, containing the α-domain, as being directly involved in the DNA-binding activity. In this work, we demonstrate that its DNA-binding properties are strongly dependent on the redox state of the α-domain active site. Site-directed mutagenesis experiments on the first cysteine residue of the –CGHC– thioredoxin-like active site lead to a mutant domain (C406S) lacking DNA-binding activity. Biochemical studies on the recombinant domain revealed a conformational change associated with the redox-dependent formation of a homodimer, having two disulfide bridges between the cysteine residues of two α-domain active sites. The formation of intermolecular disulfide bridges rather than intramolecular oxidation of active site cysteines is important to generate species with DNA-binding properties. Thus, in the absence of any dedicated motif within the protein sequence, this structural rearrangement might be responsible for the DNA-binding properties of the C-terminal domain. Moreover, NADH-dependent thioredoxin reductase is active on intermolecular disulfides of the α-domain, allowing the control of dimeric protein content as well as its DNA-binding activity. A similar behavior was also observed for whole ERp57.

ERp57, also known as ERp60, ERp61, or GRP58, is a member of the protein-disulfide isomerases family, with PDI2 as the best-known example (1, 2). These proteins present one or more domains with sequence homology to thioredoxin (3, 4). As for PDI, ERp57 consists of four domains named a, b, b’, and α’, with a and α’ containing the thioredoxin-like active site sequence WCGHCK, whereas domains b and b’ are redox inactive (4–6). ERp57 shows a ubiquitous distribution in a wide variety of cell types. It is mainly localized in the endoplasmic reticulum (ER) and shares with other family members a thiol-disulfide exchange activity strictly related to the redox properties of its thioredoxin-like active sites (2, 7–9). ERp57 and PDI, although similar in overall domain architecture and active-site motifs, have different functions that are the result of the specialized substrate binding properties, mainly localized at the level of C-terminal domains b’ and α’ (10–12). ERp57 is involved in the correct folding and disulfide bond rearrangement of misfolded glycoproteins (13, 14), requiring for this function the formation of a complex with either calnexin or calreticulin (11, 15–17). ERp57 chaperone activity plays also a role during the assembly of the major histocompatibility complex 1 and CD1d heavy chain (18, 19).

ERp57 has lately been subjected to intensive studies regarding its structural organization and function as a molecular chaperone within the ER (20–22). Preliminary studies have also indicated other functions and localizations for this protein (23). Although not associated to a direct experimental observation, ERp57 occurrence in the nucleus was derived from its isolation from the internal nuclear matrix of chicken hepatocytes (24), from nuclei of spermatids and spermatozoa (25) as well as from the nuclear fraction of lymphocytes and monocytes (26). Furthermore, ERp57 was found complexed to DNA in a variety of mammalian cell nuclei by the use of in vivo cross-linking agents (acting on intact cultured cells) such as UV radiation, cis-platinum (27), and formaldehyde (28). In addition, ERp57 was found in a multiprotein nuclear complex, also containing high mobility group proteins B1/B2, involved in the recognition of damaged DNA (29). ERp57 has been found in the cytosol, associated with nuclear protein STAT3 in HepB2 cells (30) and with the thiazide-sensitive sodium chloride cotransporter (31). Its presence in the cytosol provides further evidence that this protein can escape, in a still unknown way, from ER. In this case, its import into the nucleus may be related to the occurrence of a nuclear localization signal at the protein C-terminal region.

ERp57 showed in vitro DNA-binding properties that are strongly dependent on the protein redox state, the oxidized form being the competent one (27, 32). A study on intact recombinant protein and several deletion mutants, all obtained as glutathione S-transferase-fused products expressed in Esch-
erichia coli, led us to identify the C-terminal a’ domain as being responsible for ERp57 DNA-binding activity (33).

Today, the molecular basis of ERp57-DNA interaction remains unknown, as well as its correlation with the reductase activity and other biological functions. To gain a more detailed understanding of the biological role of ERp57, the C-terminal domain and a site-specific mutant were expressed and investigated for their biochemical and spectroscopic properties, in relation to structural changes induced by different redox states. This study provides information suggesting ERp57-specific functions within the nuclear compartment in relation with its reduct activity and DNA-binding properties.

**EXPERIMENTAL PROCEDURES**

Cloning, Expression, and Purification of the C-terminal Domain of ERp57—The coding sequence of the ERp57 C-terminal region (residues 377–505) containing the a’ domain was amplified from a plasmid containing the full-length cdNA of human ERp57, as previously described (33). Reaction was performed using two synthetic primers flanking the domain coding region. The forward primer P1 (CCGGATCCCTGGAGTTGAGTTGAGTTGAGTTGAG) contained a BamHI site; the reverse primer P2 (CCGAATTCTGCTTTAGAGATCCTCCTGTGC) contained an EcoRI site. PCR products were resolved by agarose gel electrophoresis and the amplified DNA fragment was purified using the QIAquick Gel Extraction Kit (Qiagen), digested with BamHI and EcoRI, and then ligated into BamHI and EcoRI sites of the pET29a vector (Novagen). Cloning was performed by standard procedures (34) and, after confirmation by sequencing, the protein was expressed in BL21 E. coli.

Transformed cells were grown in 2YT medium containing 0.03 mg/ml kanamycin at 30 °C with shaking until the A600 reached 0.6 OD. Cell induction was obtained with 1 mM isopropyl-1-thio-β-D-galactopyranoside, at 30 °C, for 3 h. Cells were harvested by centrifugation and resuspended in NEN buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 20 mM NaCl, 1% (w/v) SDS). Decrystallization and cleared by centrifugation at 10,000 g for 10 min. The supernatant was fractionated by ammonium sulfate precipitation. The fraction from 30 to 75% saturation was redissolved in 20 mM Tris-HCl, pH 8.0, 40 mM NaCl and dialyzed against the same buffer. Proteins were loaded onto a MacroPrep Q column (Bio-Rad) equilibrated in 20 mM Tris-HCl, pH 8.0, 40 mM NaCl and eluted with the same buffer. The flow-through fraction was collected and loaded onto a heparin column (AffiPrep Heparin, Bio-Rad) eluted with 15 volumes of a linear 40–1000 mM NaCl gradient in 20 mM Tris-HCl, pH 8.0. Fractions containing the recombinant protein were pooled and dialyzed against 10 mM Tris-HCl, pH 8.0, 10 mM NaCl. The protein was further purified on a heparin column using a narrow NaCl gradient, dialyzed, and finally concentrated by using a Vivaspin concentrator (VivaScience). The residue numbers of ERp57 here reported refer to the human cdNA sequence, including the N-terminal signal peptide. Recombinant protein contained a N-terminal pre-sequence coded by the pET29 vector, useful for obtaining fully soluble products.

Site-directed Mutagenesis on the C-terminal Domain of ERp57—The ERp57 C-terminal domain was subjected to site-directed mutagenesis to convert the first cysteine residue of the thioredoxin-like active site in a serine one (C406S mutant). Preparation of the C406S mutant was performed by overlap extension (35), using three PCR and four primers. Primers P1 and P2 were the same as reported above, whereas primers P3 (GCCCTTGGCTGTTGTCATTGG) and P4 (CTTACAGTGACAGGACGGG) were complementary, overlapping the mutation site and containing the base substitution. At first, two different PCR were used to amplify the pSVL-ERp57 vector producing two DNA fragments partially overlapping. Fragment A, corresponding to the coding region of the C-terminal domain from the beginning to the mutation site, was amplified by using primers P1 and P4. Fragment B, corresponding to the coding region of the C-terminal domain from the mutation site to the C-terminal end, was amplified by using primers P2 and P3. Amplified DNA fragments were purified and used in a third PCR using primers P1 and P2 to produce the DNA fragment C coding for the mutated C-terminal domain. Amplified DNA was digested with BamHI and EcoRI and then ligated into BamHI and EcoRI sites of the pET29a vector. Mutation was confirmed by DNA sequencing. All PCR were performed with a proofreading enzyme (AqquTaq La Polymerase, Sigma) and for a limited number of amplification cycles. Protein corresponding to the C406S mutant contained, as the wild type domain, a N-terminal pre-sequence coded by the pET29 vector and was expressed and purified as described above.

Electrophoretic Mobility Shift Assay—Mobility shift assay was performed with a 79-bp AT-rich DNA fragment, obtained by PCR as previously described (33), and with a 34-bp AT-rich DNA (GGGATCCCTTTTTAAAATTGGAAAAATTTGGATCCC) obtained by annealing two complementary synthetic oligonucleotides. DNA binding was performed in a 20-μl incubation mixture containing 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10% (v/v) glycerol, 100 ng of DNA and different amounts of protein. Binding mixtures were incubated for 30 min at 25 °C and then resolved on 5% polyacrylamide gels in TBE buffer (22.5 mM Tris borate, 5 mM EDTA, pH 8.3). After electrophoretic separation, performed at 180 V, the gels were stained for 30 min in TBE buffer containing 1 μg/ml ethidium bromide and images were recorded by a Kodak Image System equipped with a 304-nm UV lamp.

Quantification of Free Thiol Groups—Analysis of free thiol groups in ERp57 was carried out by Ellmans reagent (36). A 50-μl aliquot of protein (1 mg/ml concentration) was added to 1 ml of 150 μM 5,5’-dithiobis-2-nitrobenzoic acid in 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 20 mM NaCl, 1% (v/v) SDS. After incubation for 30 min at room temperature, the absorbance at 412 nm was recorded; the amount of free thiol groups was determined using a fresh solution of reduced glutathione to generate a standard curve.

Alkylation of Protein Samples with Iodoacetamide—Wild type C-terminal domain and C406S mutant (200 μg) were denatured in 6 M guanidine chloride, 0.25 M Tris-HCl, 1.25 mM EDTA, pH 7.0, in the absence of reducing agents, and quickly alkylated by the addition of iodoacetamide (1.1 M final concentration) at room temperature for 1 min, in the dark (37).
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Protein samples were freed from salt and reagent excess by passing the reaction mixture through an analytical Vydac C₄ column using an isocratic elution of 0.1% (v/v) trifluoroacetic acid for 35 min and then a linear gradient from 0 to 60% (v/v) of acetonitrile in 0.1% (v/v) trifluoroacetic acid over a period of 45 min at a flow rate of 1 ml/min. Protein samples were manually collected and freeze-dried.

Enzymatic Hydrolysis and Peptide Purification—Trypsin digestion was performed by incubating carboxyamidomethylated wild type C-terminal domain and C406S mutant samples (100 µg) at 37 °C overnight in 0.4% ammonium bicarbonate, pH 8.0, at a enzyme/substrate ratio of 1:100. Aliquots of the digest were directly separated on a narrow bore Vydac C₁₈ column using a linear gradient from 5 to 70% (v/v) of acetonitrile containing 0.1% (v/v) trifluoroacetic acid, over a period of 65 min, at a flow rate of 0.2 ml/min. Peptides were manually collected and further analyzed.

Mass Spectrometry—Accurate molecular mass of protein and peptide samples was measured by MALDI-TOF mass spectrometry using a Voyager DE MALDI-TOF instrument (Applied Biosystems) in a linear mode. Samples were loaded on the instrument target, using the dried droplet techniques and α-cyano-4-hydroxycinnamic acid as matrix. Mass calibration was performed using the molecular ions from different internal standards added to each sample. Data were reported as average values. Peptides were identified within the ERp57 sequence on the basis of their molecular mass and enzyme specificity.

Protein Sequencing—Automated N-terminal degradation of the purified peptides was performed using a Procise 491 protein sequencer (Applied Biosystems) equipped with a 140C microgradient apparatus and a 785A UV detector (Applied Biosystems) for the automated identification of phenylthiohydantoin-derivatives. Cystine-containing peptides were analyzed according to Ref. 38.

Spectroscopic Techniques—All spectroscopic measurements were performed with a solution of the C-terminal domain initially reduced with 25 mM DTT, for 30 min at room temperature, and then re-oxidized throughout prolonged dialysis either in 5 mM Mes, 10 mM NaCl, pH 6.5 (buffer at pH 6.5), or 5 mM Hepes, 10 mM NaCl, pH 8.0 (buffer at pH 8.0). The C406S mutant was analyzed after dialysis in buffer at pH 6.5 and 8.0.

Intrinsic fluorescence emission and light scattering measurements were carried out with a LS50B PerkinElmer spectrofluorimeter (PerkinElmer Life Sciences), using a 1-cm path length quartz cuvette. Fluorescence emission spectra (300 – 450 nm and 1-nm sampling interval) were recorded at 20 °C and at a protein concentration of 0.12 mg/ml, with the excitation wavelength set at 295 nm. Light scattering was measured at 20 °C with both excitation and emission wavelengths set at 480 nm.

Far UV CD spectra of the C-terminal domain and C406S mutant were recorded in a 0.1-cm quartz cuvette at 0.12 mg/ml protein concentration (200–250 nm) and in a 0.01-cm quartz cuvette at 1.5 mg/ml protein concentration (190–250 nm). Measurements were performed in both conditions to check if protein concentrations could have an effect on the dichroic activity; no differences were observed in the 200–250-nm region and only the spectra recorded in the 0.01-cm cuvette were reported. Near UV CD (250 –350 nm) spectra of the C-terminal domain and the C406S mutant were recorded in a 1.0-cm quartz cuvette at 1.5 mg/ml protein concentration. All CD spectra were recorded at 20 °C on a Jasco J-720 spectropolarimeter. The results are expressed as the mean residue ellipticity assuming a mean residue weight of 110 per amino acid residue. In all the spectroscopic measurements 100 µM EDTA was present unless otherwise stated. UV-visible spectra were recorded with a double-beam Lambda 16 PerkinElmer spectrometer equipped with a Peltier thermocontroller set at 20 °C.

Experiments with the fluorescent dye anilinonaphthalene-8-sulfonic acid (ANS) were performed at 20 °C by adding ANS (50 µM) to a solution of the C-terminal domain (0.08 mg/ml) reduced and then re-oxidized either in buffer at pH 6.5 or 8.0, and to a solution of the C406S mutant (0.08 mg/ml). After 5 min, fluorescence emission spectra (400 – 600 nm) were recorded with the excitation wavelength set at 390 nm. Maximum fluorescence emission wavelength and intensity of the hydrophobic probe ANS depend on the environmental polarity, e.g. on the hydrophobicity of protein accessible surface (39).

Fluorescence quenching was carried out by adding increasing amounts of acrylamide (0 – 100 mM) to a solution of the C-terminal domain (0.1 mg/ml) re-oxidized either in buffer at pH 6.5 or 8.0. Emission spectra (300 – 450 nm) were recorded at 20 °C, 10 min after each acrylamide addition with the excitation wavelength set at 290 nm. The effective quenching constants were obtained from modified Stern-Volmer plots by analyzing $F_0/\Delta F$ versus $1/[\text{acrylamide}]$ (25 data points) (40).

Cloning, Expression, and Purification of Human ERp57—The DNA fragment coding for mature human ERp57, devoid of the N-terminal pre-sequence (residues 1–24) and obtained by BamHI and EcoRI digestion of the pGEX-2T-ERp57 plasmid (33), was inserted into the BamHI and EcoRI sites of pTriEx-1.1 vector (Novagen) giving the pTriEx-1.1-ERp57 vector. The protein was expressed in BL21 E. coli and purified as previously described (41). Mature ERp57 was also purified from pig liver (32).

Transfection and Overexpression of ERp57—For the mammalian expression of fusion protein GFP-ERp57, the plasmid pTriEx-GFP-ERp57 was constructed by inserting the GFP coding DNA sequence into the BamHI restriction site of pTriEx-1.1-ERp57 vector. Cloning was performed with the procedure previously described (41). HeLa cells were transfected with pTriEx-GFP-ERp57 and cultured for 4 weeks in the presence of G418 to produce stable transfectant cell lines (41).

Cells grown on coverslips were washed in phosphate-buffered saline, fixed in 4% (w/v) formaldehyde, and mounted in 90% (w/v) glycerol containing 1 mg/ml p-phenylenediamine. Cells were examined under an epifluorescence Zeiss Axiopt microscope equipped with a CCD camera and an Argus-20 image processor (Hamamatsu). Confocal images were taken under a TCS-SP2 (Leica) microscope using a 488-nm laser line for excitation and a 500 – 537-nm band pass filter for emission. Emission signals were collected using a ×40 (NA = 1.25) or ×100 (NA = 1.4) objective.

Cells were subjected to subcellular fractionation. Cytosol and the fraction containing membrane proteins and proteins from the lumen of organelles (e.g. the ER and mitochondria) were obtained with the Qproteome Cell Compartment Kit (Qiagen),
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Whereas nuclear lysates were obtained as previously described (41). Subcellular fractions were resolved by SDS-PAGE and analyzed by Western blotting with anti-GFP antibody (Santa Cruz Biotechnology). Membranes were developed by chemiluminescence with ECL substrates (Amersham Biosciences) and subjected to quantitative analysis with a Kodak Image Station 2000R.

ERp57 overexpressing HeLa cells were also analyzed for their susceptibility to H$_2$O$_2$-induced cell killing. HeLa wild type and stably transfected cells were plated and, after incubation for 24 h, 0.5 or 2.5 mM H$_2$O$_2$ was added to the medium. The cultures were maintained for an additional 24 h and the cells surviving after incubation were examined.

Other Procedures—Sedimentation velocity experiments were carried out on the C-terminal domain reduced and re-oxidized in buffer at pH 6.5 or buffer at pH 8.0, and on C406S mutant analyzed in buffer at pH 6.5. All protein solutions were diluted to 0.8 mg/ml protein concentration. Ultracentrifugation runs were performed at 40,000 rpm and 20 °C for 4 h using a Beckman Optima XL-A analytical ultracentrifuge equipped with absorbance optics and an An60-Ti rotor. The concentration of protein was determined from absorbance at 280 nm by using the molar extinction coefficient calculated by the method of Gill and von Hippel (42). Proteins were analyzed by SDS-PAGE under reducing or non-reducing conditions by diluting samples with a 4-fold concentrated SDS sample buffer containing or not 100 mM DTT. Samples were added with 10 mM N-ethylmaleimide before dilution with concentrated SDS-sample buffer to prevent any reactivity of free thiol groups. ERp57 and the C-terminal domain were subjected to enzymatic reduction with rat liver thioredoxin reductase (Sigma). Samples (1 mg/ml) were incubated with thioredoxin reductase (0.05 mg/ml), 1.2 mM NADPH for 30 min, at 37 °C, in 20 mM Hepes, 20 mM NaCl, pH 8.0. Control samples were incubated in the absence of both thioredoxin reductase and NADPH, with thioredoxin reductase or NADPH separately, and with 25 mM DTT. Aliquots were analyzed by SDS-PAGE under non-reducing conditions and EMSA.

RESULTS

Expression and DNA-binding Properties of ERp57 C-terminal Domain and Its C406S Mutant—Previous work on the glutathione S-transferase-fused intact protein and several deletion mutants showed the importance of the a’ domain for the DNA-binding properties of ERp57 (33). The glutathione S-transferase-fused a’ domain showed a higher affinity toward DNA and confirmed the importance of the redox conditions for an effective DNA binding. To provide additional evidence of these observations and to determine the role in DNA binding control of the two cysteine residues present in the -CGHC- thioredoxin-like active site, we expressed in E. coli the a’ domain and its C406S mutant without glutathione S-transferase tagging. The region corresponding to residues 377–505 was selected mainly on the basis of sequence homology analysis with PDI and previous reports on ERp57 (4–6, 20, 21). It included the a’ domain and the protein C-terminal tail. The latter is a short but important ERp57 region containing the ER retention signal and the nuclear localization sequence. To better define biochemical properties related to the isolated a’ domain, we tried to express it without the C-terminal tail; however, these attempts did not yield satisfactory expression in our system. Cysteine 406 was selected on the basis of its estimated higher reactivity, in accordance with the observed pK$_a$ values of cysteines present in the PDI active site (4), and the protein expressed does not contain cysteine residues other than those present in the active site. SDS-PAGE analysis under reducing conditions of the purified C-terminal domain and its C406S mutant is shown in Fig. 1A. Mass spectrometric investigation on reduced and carboxymethylated samples confirmed the expected sequence for both recombinant products (C-terminal domain: measured MH$^+$ at m/z 18399.5; theoretical MH$^+$ at m/z 18400.0; C406S mutant: measured MH$^+$ at m/z 18326.2; theoretical MH$^+$ at m/z 18326.9).

Wild type C-terminal domain and C406S mutant were tested for their DNA binding capability by means of EMSA. Previous results obtained by DNA-binding assays performed in vitro indicated an Erp57 preference toward AT-rich DNA; no significant evidence on protein binding to a specific DNA sequence was observed. To better define DNA properties necessary to observe optimal binding, DNA fragments of different sizes containing AATT and TTAA stretches were compared by EMSA (Fig. 1B). We observed that interaction occurred only with AT-rich DNA fragments longer than 30 bp, suggesting that DNA binding is mainly based on a particular DNA conformation rather than a specific sequence. A similar behavior has been observed for HMG1/2 proteins, which preferentially bind to AT-rich DNA sequences presenting a bent structure (43). Thus, we used a 34-bp AT-rich DNA fragment for the following experiments. The wild type C-terminal domain displayed a high affinity toward this DNA fragment with the formation of complexes even at a protein/DNA molar ratio lower than 100 (Fig. 1C). Because of the higher binding properties of the C-terminal domain compared with the whole ERp57, we were able to perform EMSA analysis by simply revealing DNA-protein complexes by ethidium bromide staining. The presence of 10 mM

FIGURE 1. Analysis of the purified ERp57 C-terminal domain and C406S mutant. A, SDS-PAGE analysis under reducing conditions of the purified wild type domain and C406S mutant expressed in E. coli. Each lane was loaded with 3 μg of protein. Migration of molecular mass markers is also indicated. B, EMSA analysis of the domain (5 μg of protein) in the presence of different AT-rich DNA fragments (150 ng each) containing AATT and TTAA stretches and increasing length (16 bp, GGATAAAATTTGGATCC; 20 bp, GGATCATAAAATTTGGATCC; 24 bp, GGATCTTAAATTTGGATCC; 28 bp, GGATCTTTAAATTTGGATCC; 32 bp, GGATCTTTAAATTTGGATCC). C, EMSA analysis of the wild type domain and C406S mutant. The assay was performed in the presence of 75 ng of AT-rich 34-bp DNA and increasing amounts of protein.
DTT fully abolished the binding activity of the C-terminal domain, indicating the importance of the protein redox state for an effective binding to DNA and suggesting a specific role for the thioredoxin-like active site. This was confirmed by comparative analysis of the wild type domain and C406S mutant. In fact, EMSA analysis performed under the same experimental conditions showed mutated domains as incompetent for DNA binding (Fig. 1C).

**Structural Analysis of Wild Type C-terminal Domain and C406S Mutant**—To investigate the oxidation state of the cysteine residues, the purified C-terminal domain and C406S mutant were characterized for their sensitivity to reducing conditions. SDS- and native-PAGE analysis, performed in the absence of reducing agents, revealed that the purified recombinant wild type domain existed as a heterogeneous mixture, with dimer being the most representative species (about 70%) (Fig. 2A). Dimer molecule disappeared when the sample was analyzed in the presence of DTT, indicating that dimerization was due to disulfide bridges between different polypeptides. Reaction of free thiol groups after protein denaturation was prevented by performing SDS-PAGE analysis on samples added with 10 mM N-ethylmaleimide before dilution with concentrated SDS sample buffer. In contrast, the C406S mutant analyzed under the same experimental conditions migrated always as a single monomeric form. To investigate the molecular structure of the species reported in Fig. 2A, wild type domain and C406S mutant samples were alkylated with iodoacetamide under denaturing non-reducing conditions, desalted, and separated by RP-HPLC. The carboxyamidomethyl groups introduced with the quenching reaction increased the molecular mass of the different species by a fixed value (57 Da for each free SH group), at the same time blocking the reactivity of the remaining cysteine residues. This allowed the separation by mass of species containing a different number of inter- and intramolecular disulfide bonds (44). As expected on the basis of the SDS-PAGE analysis, the chromatographic profile of the wild type domain showed the occurrence of two distinct components (Fig. 2B). On the other hand, a single molecular species was observed in the case of the C406S mutant (Fig. 2C). Mass spectrometry analysis of the less retained component present in Fig. 2B showed the occurrence of a single molecular species with a mass value corresponding to a monomeric C-terminal domain having an intramolecular disulfide (theoretical MH$^+$ at m/z 18283.9) (Fig. 2D). Similarly, the spectrum of the second component (Fig. 2B) also showed a single species whose molecular mass was associated to a homodimeric C-terminal domain form containing two intermolecular disulfides (theoretical MH$^+$ at m/z 36566.8) (Fig. 2E). This result demonstrated the occurrence of the molecular species fully oxidized in the wild type domain sample. Mass spectrometry analysis of the C406S mutant showed the presence of a single protein component with a mass corresponding to a monomeric C406S mutant bearing a carboxyamidomethyl group (theoretical MH$^+$ at m/z 18326.9) (Fig. 2F). This result demonstrated the presence of a fully reduced species in the C406S mutant, thus suggesting that the occurrence of a cysteine at position 406 promotes exhaustive protein oxidation.

To identify the nature of the observed disulfides, all purified recombinant species were digested with trypsin. The peptide mixtures obtained were resolved in parallel by RP-HPLC in almost 20 elution peaks and each peptide component was further characterized by MALDI-TOF-MS and Edman degradation. MS analysis allowed the determination of the redox state of the cysteine residues present in each peptide. In fact, the occurrence of S-S bonds was confirmed by a reduction of the sample with DTT followed by MS identification of the reduced species whose molecular mass was associated to a homodimeric C-terminal domain form containing two intermolecular disulfides (theoretical MH$^+$ at m/z 36566.8) (Fig. 2E). This result demonstrated the occurrence of the molecular species fully oxidized in the wild type domain sample. Mass spectrometry analysis of the C406S mutant showed the presence of a single protein component with a mass corresponding to a monomeric C406S mutant bearing a carboxyamidomethyl group (theoretical MH$^+$ at m/z 18326.9) (Fig. 2F). This result demonstrated the presence of a fully reduced species in the C406S mutant, thus suggesting that the occurrence of a cysteine at position 406 promotes exhaustive protein oxidation.

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intramolecular Cys406–Cys409 disulfide. In the case of dimeric nature of this species, demonstrating the occurrence of the analysis during Edman degradation analysis (38) confirmed the occurrence of an intramolecular S-S bridge. Phenylthiohydantoin-Cys-Cys 1780.3, which was assigned to peptide-(396–410) containing an intramolecular S-S was absent. On the contrary, a peak at 51 min containing two intermolecular disulfides, the peptide-(396–410) linked by two disulfide bridges. Edman degradation confirmed this hypothesis and suggested the occurrence of two identical intermolecular peptides linking Cys406–Cys409. As expected, the tryptic digest of the monomeric C406S mutant showed the absence of the above mentioned peaks and the presence of a unique peak at 29 min. MALDI-TOF-MS analysis of this fraction demonstrated the occurrence of a single peptide species with a MH+ at m/z 1823.3 that was assigned to the carboxamidomethylated peptide-(396–410). Edman degradation analysis demonstrated the occurrence of Ser and Cys-CAM at positions 406 and 409, respectively.

DNA binding capability of the C-terminal domain was tested in the presence of increasing amounts of DTT. EMSA analysis showed a promptly loss in DNA-binding activity at a DTT concentration above 0.2 mM (Fig. 3A). Native-PAGE analysis, performed in the same range of DTT concentration, revealed a parallel variation in the mobility of the C-terminal domain (Fig. 3B) confirming that its DNA binding capability is based on a conformation that involves the redox state of cysteine residues.

**Dimeric Wild Type C-terminal Domain Is Responsible for Binding DNA**—To control the nature of the oxidized species in the C-terminal domain preparations and their ability to bind DNA, we attempted to re-oxidize the recombinant product in buffers having a different pH value. These conditions could have an effect on the capability of thiols to form intra- or intermolecular disulfide bridges. Purified C-terminal domain was fully reduced by treatment with 25 mM DTT in 20 mM Tris-HCl, pH 8.0, 50 mM NaCl, for 30 min, at room temperature and then allowed to re-oxidize throughout prolonged dialysis in different buffers without reducing agents (10 mM Mes, pH 6.5, 10 mM Hepes, pH 7.0, pH 7.5 and 8.0, all containing 20 mM NaCl). Ellmans determination was performed during dialysis to test the redox state of cysteine residues, confirming the complete oxidation of protein samples after 48–96 h (data not shown). Aliquots of the domain preparations re-oxidized at different pH values were then analyzed by SDS-PAGE in the presence or absence of DTT in the sample buffer. As shown in Fig. 4A, the amount of the dimer stabilized by intermolecular disulfides bridges significantly decreased lowering the buffer pH value, and an essentially homogenous solution of monomeric domain was observed at pH 6.5. Chromatographic and mass spectrometry investigations, following exhaustive alkylation under denaturing non-reducing conditions of all resulting products confirmed the observed monomer/dimer ratios and a complete oxidized state for the analyzed species. Aliquots of the C-terminal domain re-oxidized at different pH values were also analyzed by EMSA to test their DNA-binding properties. EMSA analysis revealed a decrease in the amount of protein-DNA complexes when lowering the pH value from 8.0 to 6.5 (Fig. 4B), which correlates with the amount of dimeric domain observed (Fig. 4A). DNA-binding activity was not affected by the intrinsic...
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pH value during the assay because a preparation of the C-terminal domain re-oxidized at pH 8.0, diluted in a buffer at pH 6.5 and then immediately tested by EMSA, did not show any significant variation in its ability to generate protein-DNA complexes (data not shown). These results indicated that the dimerization state induced by Cys406 reactivity is more important than the redox state of the CHGC-active site for the DNA-binding properties of the domain.

To separate monomeric and dimeric forms, the C-terminal domain was fully reduced and then separately re-oxidized in buffer at pH 6.5 and 8.0. Proteins were tested for their redox state by Ellman's determination and SDS-PAGE analysis under non-reducing conditions, and for their DNA-binding activity. The results obtained were in agreement with those reported above and in Fig. 4. The domain oxidized at pH 6.5 showed a monomeric form in non-reducing SDSPAGE analysis and no DNA-binding activity, whereas the same protein oxidized at pH 8.0 was mainly composed of dimeric species and showed DNA-binding activity. The results on the protein association state obtained by SDS-PAGE found a positive verification by a sedimentation velocity assay. In fact, the domain oxidized at pH 6.5 and the C406S mutated domain showed a single sedimentation species corresponding to the monomeric form, whereas the domain oxidized at pH 8.0 occurred as a mixture containing almost 70% of dimeric species (Fig. 5). The fast sedimentation species present in the domain oxidized at pH 8.0 disappeared after reduction with 20-fold molar excess DTT, thus indicating that dimerization of the C-terminal domain is promoted and stabilized by the formation of inter-molecular disulfide bridges.

Analysis of Monomer-Dimer Conformational Change—To detect any structural rearrangement caused by variation in the redox and dimerization state and possibly related to the different DNA-binding activities, the spectroscopic properties of recombinant wild type C-terminal domain and C406S mutant were investigated (Fig. 6). Monomeric wild type domain, obtained after reduction and re-oxidation in buffer at pH 6.5, was subjected to spectroscopic analysis and compared with the same domain re-oxidized in buffer at pH 8.0 (mainly corresponding to the dimeric species), and to the C406S mutant, which under the same experimental conditions always occurred in a reduced-monomeric form. The far-UV CD spectrum of the domain oxidized at pH 6.5 (Fig. 6A) was typical of a α/β protein and identical to that of the C406S mutant analyzed under the same conditions (Fig. 6B). The near-UV CD spectrum of the domain oxidized at pH 6.5 was characterized by distinct bands corresponding to putative contribution of Phe (261 and 268 nm), Tyr (277 nm), and Trp (285 nm) (Fig. 6C), and by a broad positive ellipticity signal around 300 nm possibly ascribed to the effect of disulfide as chromophore (45, 46). The near-UV CD spectrum of the C406S mutant obtained at the same pH value showed similar aromatic contributions, but was characterized by an increased dichroic activity and the absence of the positive band at around 300 nm (Fig. 6D). The far UV CD spectrum of the domain oxidized at pH 8.0 changed dramatically when compared with that oxidized at pH 6.5; its zero-intercept was 3 nm blue-shifted and centered at 196 nm, and the θ222/θ208 ratio decreased from 0.81 to 0.66 (Fig. 6A). Moreover, the dichroic activity of the domain oxidized at pH 8.0 in the near UV CD region was decreased in all the aromatic spectral components; the 300-nm band was broadened and shifted to a longer wavelength (Fig. 6C). The addition of 20-fold molar excess DTT to the domain oxidized at pH 8.0 induced dramatic changes in the far UV CD spectrum; the zero intercept was red-shifted to 199 nm (Fig. 6A) and the θ222/θ208 ratio increased. Upon addition of DTT to the domain oxidized at pH 8.0 all the aromatic contributions in the near UV CD spectrum became more distinct and the broad band around 310 nm disappeared (Fig. 6C). Therefore, the near and far UV CD spectra of the wild type domain at pH 8.0 in the presence of the reducing agent (Fig. 6A and C) were closely similar to those of the C406S mutant monitored under the same conditions (Fig. 6B and D). CD spectra of the C406S mutant obtained at pH 8.0 and 6.5 were similar and the addition of DTT did not induce any significant changes (Fig. 6, B and D).

The intrinsic fluorescence emission spectrum of the domain oxidized in buffer at pH 8.0, measured upon excitation at 295 nm, had a maximum emission wavelength centered at 346 nm. The presence of 20-fold molar excess DTT induced a 31% decrease of the fluorescence intensity and a maximum emission wavelength at 347 nm (Fig. 6E). On the contrary, the intrinsic fluorescence emission intensity of the domain oxidized in buffer at pH 6.5 was 50% lower than that measured for the protein oxidized at pH 8.0, with a maximum emission wavelength at 349 nm (Fig. 6E). Intrinsic fluorescence emission spectra of the C406S mutant were measured at pH 6.5 and 8.0, in the absence or presence of DTT, showed intensities closely similar to that of the domain oxidized at pH 6.5, and maximum emission wavelengths centered, respectively, at 346 and 348 nm (Fig. 6, E and F). Light scattering measurements indicated that aggregation of the wild type domain and the C406S mutant never occurred in all the conditions tested.

All the above mentioned results indicate a marked conformational change affecting the C-terminal domain upon dimerization through the formation of intermolecular disulfide bridges. This conformational change is not the result of cysteine redox state variation because the domain oxidized in buffer at pH 6.5, which is in a monomeric form with an intramolecular disulfide bridge, did not show significant differences in the near and far UV CD spectra and intrinsic fluorescence compared with the C406S mutant.
Accessibility of hydrophobic residues in domain samples oxidized in buffer at pH 8.0 and 6.5 was evaluated by means of the fluorescent probe ANS. Comparative analysis of ANS emission fluorescence spectra showed an increase in intensity in the presence of the protein oxidized at pH 8.0, and a blue shift from 513 to 478 nm (Fig. 7). On the contrary, the ANS emission fluorescence spectrum was not affected by experimental pH and did not show any significant variation in the presence of the domain oxidized at pH 6.5 or the C406S mutant (Fig. 7). These results suggest that dimer formation is accompanied by an increase of the hydrophobic surface accessible on the protein.

Sensitivity of the Intermolecular S-S Bridges to Thioredoxin Reductase Activity—We recently evidenced that ERp57 is a substrate of NADPH-dependent thioredoxin reductase activity and disulfides present in the oxidized ERp57 can be reduced to free thiol residues as result of the enzymatic reaction (41). To test the sensitivity of intermolecular disulfide bridges to NADPH-dependent thioredoxin reductase activity, the oxidized-dimeric C-terminal domain was subjected to enzymatic reduction. SDS-PAGE analysis performed under non-reducing conditions showed a loss of dimeric species after enzymatic treatment (Fig. 8A). Likewise, enzymatic treatment strongly reduced DNA-binding activity of the C-terminal domain as revealed by EMSA (Fig. 8B). Neither thioredoxin reductase nor NADPH separately produced the same effect. These data indicated that the intermolecular disulfide bridges stabilizing the dimeric form of the C-terminal domain are substrates of the NADPH-dependent thioredoxin reductase.

Intermolecular S-S Bridges in ERp57 and Their Sensitivity to Thioredoxin Reductase Activity—The occurrence of intermolecular disulfide bridges was also investigated on whole ERp57. ERp57 was purified from pig liver and subjected to SDS-PAGE analysis under reducing and non-reducing conditions. Electrophoretic analysis showed that even ERp57 might exist as a homodimer stabilized by intermolecular disulfide bridges, which disappear on reduction with DTT (Fig. 9A). A similar result was obtained analyzing the recombinant human ERp57. Dimerization status and

The conformational change between monomeric and dimeric forms was also supported by comparative experiments on C-terminal domain samples oxidized in buffer at pH 6.5 and 8.0. Accessibility and dynamic properties of domain samples were studied by means of protein intrinsic emission fluorescence quenching with acrylamide (data not shown). The quenching constants of the protein oxidized at pH 8.0 and 6.5, obtained by Stern-Volmer graphs, were, respectively, 10.6 M\(^{-1}\) and 4.4 M\(^{-1}\). These data indicated that the dimeric form of the C-terminal domain presented an increase in molecular surface exposed to collisional quenching.
thus providing a justification to the nuclear signal present at the protein C-terminus. To provide further evidence of the nuclear localization of ERp57 and investigate its functions in this compartment, HeLa cells were stably transfected with an expression vector coding for a GFP-tagged ERp57. This tag has been widely used to verify the in vivo cellular distribution of other proteins. Moreover, the fusion protein does not contain the 24-residue signal peptide that normally drives ERp57 inside the ER, making this experimental system helpful to analyze ERp57 functions outside of this compartment. Cells were selected with G418 and screened using GFP for clones that stably expressed GFP-ERp57 protein. HeLa cells expressing GFP-ERp57 were subjected to subcellular fractionation and Western blot analysis using a monoclonal anti-GFP antibody. As expected, most of the fusion protein was localized in the cytosolic fraction but a detectable level was evident in the nuclear lysate (Fig. 10A). Fluorescence analysis with confocal microscopy confirmed this cellular distribution and the presence of the fusion protein associated with the nucleus (Fig. 10B).

HeLa cells overexpressing ERp57 were tested for their susceptibility to H₂O₂-induced cell killing. Previous results indicated a marked protective effect of ERp57 (41), thus confirming its important role in cellular response to oxidative stress. Western blot analysis on different subcellular fractions and microscopy fluorescence experiments showed a 2-fold increase in the nuclear localization of GFP-ERp57 following H₂O₂-induced oxidative stress (Fig. 10, C and D), suggesting specific functions for ERp57 within the nuclear compartment. Moreover, considering that the fusion protein construct is under the control of a noninducible promoter (i.e. β-actin), the comparative analysis of Western blot data suggests a cellular redistribution of GFP-ERp57 following oxidative stress, which mainly involve nuclei, membranes, and cellular organelles.

To test the presence of intermolecular S-S bridges involving nuclear ERp57, subcellular fractionation of HeLa-transfected cells was performed in the absence of reducing agents, whereas

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FIGURE 9. Presence of intermolecular S-S bridges in ERp57 and their sensitivity to thioredoxin reductase activity. A, SDS-PAGE analysis under non-reducing (−) or reducing (+) conditions of ERp57 purified from pig liver. Each lane was loaded with 2 μg of protein. Migration of molecular mass markers is also indicated. B, SDS-PAGE analysis under non-reducing conditions of ERp57 subjected to enzymatic reduction by thioredoxin reductase and NADPH. Equal amounts of proteins were incubated alone (lane 1), with thioredoxin reductase and NADPH (lane 2), with thioredoxin reductase (lane 3), with NADPH (lane 4), or with DTT (lane 5), and then resolved by 10% SDS-PAGE. Each lane was loaded with 2 μg of protein. C, aliquots of ERp57 incubated as described for B were analyzed by EMSA in the presence of 1 ng of ³²P-labeled AT-rich 34-bp DNA and a fixed amount of protein (protein/DNA = 1000:1 mol/mol).

DNA-binding activity were also tested in ERp57 subjected to enzymatic reduction by thioredoxin reductase and NADPH. As well as observed for the C-terminal domain, non-reducing SDS-PAGE and EMSA analysis showed that intermolecular disulfide bridges present in ERp57 are substrates of thioredoxin reductase and that DNA-binding capability drops following the enzymatic treatment (Fig. 9, A and B).

Nuclear Distribution of ERp57 and Protection from Oxidative Stress—Some preliminary observations indicate that ERp57 localization is not restricted to ER but also to the cell nucleus,

FIGURE 8. Sensitivity of intermolecular S-S bridges in the wild type C-terminal domain to NADPH-dependent thioredoxin reductase activity. A, SDS-PAGE analysis under non-reducing conditions of the C-terminal domain subjected to enzymatic reduction by thioredoxin reductase and NADPH. Equal amounts of proteins were incubated alone (lane 1), with thioredoxin reductase and NADPH (lane 2), with thioredoxin reductase (lane 3), with NADPH (lane 4), or with DTT (lane 5), and then resolved by 12% SDS-PAGE. Each lane was loaded with 2 μg of protein. B, aliquots of the C-terminal domain incubated as described for A were analyzed by EMSA analysis in the presence of 1 ng of ³²P-labeled AT-rich 34-bp DNA and a fixed amount of protein (protein/DNA = 1000:1 mol/mol).

FIGURE 7. Relative accessibility of hydrophobic residues in the wild type domain, reduced and then re-oxidized either at pH 6.5 or 8.0, and in the C406S mutant, as monitored by ANS extrinsic fluorescence. ANS (50 μM) was added to a solution of the wild type domain (0.08 mg/ml) reduced and then re-oxidized either in buffer at pH 6.5 (dotted line) or 8.0 (continuous line), and to a solution of the C406S mutant (0.08 mg/ml) previously incubated in buffer at pH 6.5 (dashed line). Fluorescence emission spectra (390 nm excitation wavelength) were recorded at 20 °C, 5 min after the addition of ANS.

DNA-binding activity were also tested in ERp57 subjected to enzymatic reduction by thioredoxin reductase and NADPH. As well as observed for the C-terminal domain, non-reducing SDS-PAGE and EMSA analysis showed that intermolecular disulfide bridges present in ERp57 are substrates of thioredoxin reductase and that DNA-binding capability drops following the enzymatic treatment (Fig. 9, A and B).

Nuclear Distribution of ERp57 and Protection from Oxidative Stress—Some preliminary observations indicate that ERp57 localization is not restricted to ER but also to the cell nucleus,
Comparable amount of cytosolic (C) and nuclear lysate (N) fractions, corresponding to 2 × 10^6 cells, and nuclear lysate (N), corresponding to 6 × 10^5 cells, were subjected to analysis. Migration of molecular mass markers is also indicated. 

Upper panel reports a quantitative analysis of developed membranes. Data reported are expressed in arbitrary units and are the result of three independent experiments. B and D, HeLa cells that stably express GFP-ERp57 grown on coverslips were incubated for 24 h in the absence (B) or presence (D) of 2.5 mM H_2O_2. Cells were fixed, mounted, and images were taken under a TCS-SP2 (Leica) confocal microscope. The purified domain undergoes dimerization by disulfide bond formation and the occurrence of the dimer is significantly related to the DNA-binding properties of the protein. Although ERp57 a’ domains have been previously expressed, always as His-tagged products (20, 21), its dimerization has never been reported. This different behavior may be ascribed to the different nature of the protein product, having reduced dimension, a different tag, and not containing the protein C-terminal tail. This highly charged region, namely QEEKPKKKKKAQDEL, contains the nuclear localization signal, absent in other proteins having the thioredoxin fold, which may play an important role for cellular localization, protein function, and stability. On the other hand, the isolated α’ domain of PDI, not containing this nuclear localization sequence, is marginally conformationally stable and easily unfolds as result of oxidation (47).

The data reported in this article demonstrates that the cysteine residues of the thioredoxin-like active site in the α’ domain and their particular redox state play an essential role in the control of DNA-binding activity. In fact, EMSA experiments showed that the C406S mutant did not display any interaction with DNA and that the addition of reducing agents to wild type protein fully abolished its binding properties. Parallel PAGE and MALDI-MS analyses under non-reducing conditions on the C-terminal domain and ERp57 samples that have DNA-binding activity, demonstrated the existence of dimeric species with intermolecular disulfide bonds. In the case of the C-terminal domain, the nature of these intermolecular S-S bridges was characterized by a peptide mass mapping approach, suggesting the occurrence of two identical intermolecular disulfide bridges linking Cys^406 and Cys^409. Intermolecular disulfide formation is influenced by environmental pH, which likely regulates the reactivity of cysteine residues at the active site. In the absence of such intermolecular disulfide bonds, i.e. in the reduced domain, in the domain oxidized at low pH or in the C406S mutant, proteins exist as monomeric species lacking any DNA-binding properties.

Comparative spectroscopic measurements on monomeric and homodimeric domains demonstrated that dimerization is associated with a marked conformational change. The dimeric domain retained spectroscopic signals in the near UV CD spectrum and its structural rearrangement was fully reversible upon disulfides reduction. Spectroscopic light scattering measurements showed that the dimeric protein did not aggregate, and velocity sedimentation analysis showed a peak in the sedimentation profile corresponding to the dimeric species. The folding rearrangement induced by intermolecular S-S bridge formation is essential for generating the protein form competent for DNA binding in the absence of any DNA binding motifs within the protein sequence. This structural variation is not only the result of a cysteine redox state change, because an oxidized-monomeric domain, containing an intramolecular disulfide bridge, presents a conformation similar to the C406S mutant and totally different from the dimeric domain.
The structural variation observed in the dimeric form may be explained assuming that the overall folding of the ERp57 a’ domain is similar to that of thioredoxin or PDI a’ domain, for which crystallographic and/or structural data have been reported (48, 49). In this case, formation of intermolecular disulfide bridges would be possible only assuming a distortion of the active site geometry within each monomer, thus justifying the experimentally observed conformational variations mainly responsible of the DNA-binding properties of the domain. In this view, the protein may present a reversible distorted conformation, characterized by an extended structure associated to the DNA-binding activity. The increased accessibility of hydrophobic residues to ANS upon dimerization and the fluorescence quenching data may add evidences to this view. On the other hand, the formation of intramolecular disulfide bridges led to minor conformational variations, in agreement with previous biophysical studies on the ERp57 a’ domain (21) and PDI a’ domain (48, 49). According to the data reported above, we propose a scheme of the different redox, conformational, and association states of the ERp57 C-terminal domain, which takes into account their distinctive DNA-binding properties (Fig. 11).

Although analyses have been performed on the isolated C-terminal domain, where dimerization could be facilitated by the absence of the other protein domains, this structural rearrangement can be hypothesized to occur also in whole ERp57 protein. In fact, ERp57 shows in vivo DNA-binding activity that is dependent on its redox state and in this work we were able to isolate ERp57 as a dimer stabilized by intermolecular disulfide bridges, whose concentration is related, at least in vitro, to the DNA-binding properties of the protein. ERp57 dimerization through intermolecular disulfide bridges has never been reported so far, but studies on PDI and thioredoxin have shown that these proteins can also exist as dimeric species, the latter stabilized by an intramolecular disulfide bridge not involving active site cysteine residues (3, 48, 50). On the other hand, the redox-dependent conformational change of the ERp57 C-terminal domain may be the result of intramolecular disulfide bridges involving both a and a’ domain active sites. In fact, the recently solved crystal structure of yeast PDI has revealed that the four protein domains are arranged according to a twisted “U” fold, with redox-active sites facing each other (51). Finally, intermolecular disulfide bridge may involve a’ domain active site and thiol groups of other protein components.

All these observations might not only be important for ERp57 structure-function relationship analysis, but also might raise questions on the biological relevance of this protein within the cell nucleus. Recent results have demonstrated that ERp57 binds in vivo to specific DNA target sequences, which are mainly present in intronic sequences similar to regulatory regions (52). The nature of the associated genes leads us to hypothesize the involvement of ERp57 in the regulation of stress-response gene expression. The data reported in this work on the increase of the ERp57 concentration within the nucleus after oxidative stress are in good agreement with this hypothesis. Our experiments suggest that ERp57 DNA-binding properties are dependent on a redox-dependent conformational change involving the protein a’ domain. Thus, not only the concentration but also the nature of ERp57 species competent for DNA binding in the nucleus could be significantly affected by oxidative stresses. We have also shown that the disulfide bridges stabilizing this competent form are substrates of the NADPH-dependent thioredoxin reductase. This enzymatic system, which act not only on thioredoxin but also on PDI (53), an enzyme with marked structural similarities to ERp57, could trigger ERp57-DNA interaction in vivo through the reduction of the protein. The presence of the NADPH-thioredoxin reductase system in the nucleus (54) is compatible with this hypothesis.

ERp57 has been reported as altering the formation of complexes between nuclear proteins and the regulatory domain of interferon-inducible genes (55). Similarly, it has also been demonstrated that both PDI and ERp57 have a regulatory effect on transcription factor E2A activity (56). Recently, we showed evidence for a cooperative activity of ERp57 and Ref-1 in the reductive activation of the API transcription factor (41) and hypothesized that ERp57 may replace or cooperate with thioredoxin in the Ref-1-dependent reduction and activation of transcription factors. Accordingly, ERp57 may specifically interact with other nuclear proteins that require its disulfide isomerase activity to maintain proper functionality generating macromolecular complexes whose affinity for DNA may be enhanced or regulated by the redox-dependent DNA-binding properties of ERp57.

All these data suggest a mechanism of action for ERp57 as a stress response protein that uses intrinsic redox changes to control biological activities. Further investigations are now in progress to better define the biological role of ERp57 in the nucleus.

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