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The DNA-binding activity of protein disulfide isomerase ERp57 is associated with the *a′* domain☆

Caterina Grillo, Sabina Coppari, Carlo Turano, and Fabio Altieri*

Dipartimento di Scienze Biochimiche "A. Rossi-Fanelli," Centro di Biologia Molecolare del CNR and Istituto Pasteur-Fondazione Cenci Bolognetti, Università "La Sapienza," P.le A. Moro 5, 00185 Rome, Italy

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Abstract

ERp57 belongs to the protein disulfide isomerases, a family of homologous proteins mainly localized in the endoplasmic reticulum and characterized by the presence of a thioredoxin-like folding domain. ERp57 is a protein chaperone with thiol-dependent protein disulfide isomerase and additional activities and recently it has been shown to be involved, in cooperation with calnexin or with calreticulin, in the correct folding of glycoproteins. However, we have demonstrated that the same protein is also present in the nucleus, mainly associated with the internal nuclear matrix fraction. In vitro studies have shown that ERp57 has DNA-binding properties which are strongly dependent on its redox state, the oxidized form being the competent one. A comparison study on a recombinant form of ERp57 and several deletion mutants, obtained as fusion proteins and expressed in *Escherichia coli*, allowed us to identify the C-terminal *a′* domain as directly involved in the DNA-binding activity of ERp57.

Keywords: ERp57; Protein disulfide isomerase; Protein–DNA interactions; Protein expression

The protein ERp57, also known as ERp60, ERp61, or GRP58, belongs to the protein disulfide isomerases, a family of homologous proteins with PDI as the best-known member [1,2]. Proteins of this family are mainly localized in the endoplasmic reticulum and characterized by the presence of a folding domain similar to that of thioredoxin, containing an active site with the amino acid sequence WCGHCK [3]. Their established function is to act as chaperones and disulfide-rearrangement enzymes to aid in the proper folding of newly synthesized proteins [4].

ERp57 consists of four domains named *a*, *b*, *b′*, and *a′*, with *a* and *a′* containing the thioredoxin-like active site [3]. In the past, a variety of functions have been attributed to ERp57 [5–7], but at present some questions about the real functions of this protein are still unanswered.

Reductase activity associated with ERp57 has been repeatedly reported [8]. This function is strictly related to the redox properties of the two thioredoxin-like active sites. ERp57 is mainly localized in the endoplasmic lumen and involved, through its reductase activity, in the correct folding and in the disulfide bond rearrangement of misfolded glycoproteins [9]. It has also been demonstrated that this function requires the formation of a complex between ERp57 and either calnexin or calreticulin [10,11]. The same proteins are found to be associated during the assembly of the major histocompatibility complex I [12].

Recent experimental evidence, however, has indicated other functions and localizations of ERp57. The activation of transcription factor STAT3 in the cytosol has to be accompanied by the formation of a multicomponent complex containing STAT3, ERp57, and other proteins [13]. Also nuclear STAT3 is known to form complexes with other proteins, although there is no evidence of the presence of ERp57 in these nuclear

☆ *Abbreviations:* SDS–PAGE, SDS–polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assay; PCR, polymerase chain reaction; GST, glutathione *S*-transferase; PVDF, polyvinylidene difluoride.

* Corresponding author. Fax: +39-06-444-0062.
E-mail address: fabio.altieri@uniroma1.it (F. Altieri).

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complexes. Calreticulin, the main cytosolic ERp57 interacting protein, has recently been shown to be present in the nucleus and involved in the nuclear export of some proteins [14].

We have previously demonstrated a nuclear distribution of ERp57, mainly associated with the internal nuclear matrix fraction [15]. Nuclear localization of ERp57 has also been reported in other cellular systems [16,17]. Cross-linking studies with *cis*-diamine-dichloroplatinum (*cis*-DDP) and UV-radiations confirmed the nuclear localization of ERp57 and its binding to DNA in chicken liver nuclei and intact cultured cells, preferentially within the matrix-associated regions (MARs) [18,19]. Moreover, Erp57 shows in vitro DNA-binding properties which are strongly dependent on the redox state of the protein, the oxidized form being the competent one [18,19].

At present little is known about the binding domains of ERp57, the molecular basis of such interactions and their correlation with the reductase activity, and the biological functions of the protein. Knowledge of the ERp57 DNA-binding domain will help elucidate the biological role of the protein. For this reason we performed a study on a recombinant form of ERp57 and several deletion mutants obtained as fusion proteins and expressed in *Eschericha coli*. With this approach we have been able to identify the a' domain as directly involved in the DNA-binding activity of ERp57.

Materials and methods

Purification of microsomal ERp57. ERp57 was purified from a pig liver microsomal extract. Liver homogenate was centrifuged at 5000*g* and 27,000*g* to remove nuclear and mitochondrial fractions, respectively. Microsomes were precipitated by ultracentrifugation at 105,000*g* for 60 min, resuspended in 0.1 M Tris–HCl, pH 8.0, and then extracted with 0.2% Triton X-100 for 60 min at 4 °C. The supernatant obtained after centrifugation at 105,000*g* for 60 min was fractionated by ammonium sulfate precipitation. The fraction from 30% to 70% saturation was redissolved in 20 mM Tris–HCl, pH 8.0, 20 mM NaCl and dialyzed against the same buffer. Proteins were applied on a Macro-Prep Q column (BioRad) eluted with 150 mM NaCl in 20 mM Tris–HCl, pH 8.0. This protein fraction was applied on a HTP hydroxyapatite column (BioRad) eluted with 10 volumes of a linear gradient 0–500 mM potassium phosphate buffer, pH 7.2. Fractions containing ERp57 were collected, dialyzed against 20 mM Tris–HCl, pH 8.0, 20 mM NaCl, and applied on a second Macro-Prep Q column (BioRad) eluted with 15 volumes of a linear 20–250 mM NaCl gradient in 20 mM Tris–HCl, pH 8.0. To purify ERp57 finally, selected fractions were pooled and applied on a Heparin Econo column (BioRad) eluted with 20 volumes of a linear 100–1000 mM NaCl gradient in 20 mM Tris–HCl, pH 8.0. SDS–PAGE of purified ERp57 is shown in Fig. 2.

Electrophoretic mobility shift assay (EMSA). Mobility shift assay was performed with oligonucleotide fragments having a different base composition and corresponding to the sequences 1153–1231 (79 bp, 73% AT), 2566–2638 (73 bp, 67% GC), and 2836–2915 (80 bp, 49% AT) of pSP65 plasmid (EMBL:CVPSP65, AC:X65329). DNA fragments were obtained by polymerase chain reaction (PCR) using specific primers, further purified by 15% polyacrylamide gel electrophoresis, and then end-labeled by T4 polynucleotide kinase reaction (ReadyToGo, Amersham) in the presence of 1 μCi [γ-^{33}P]ATP. DNA binding was performed in 20 μl incubation mixture containing 20 mM Tris–HCl, pH 7.5, 50 mM NaCl, 10% glycerol, 1 ng of radiolabeled DNA, and different amounts of ERp57 protein. Binding mixtures were incubated for 30′ at 16 °C and then resolved on 5% polyacrylamide gels in 0.25% TBE (22.5 mM Tris–borate, 5 mM EDTA, pH 8.3). Electrophoresis was performed at 180 V for 2.5 h at 16 °C. Gels were vacuum-dried and exposed overnight to a Kodak BioMax XS film.

Cloning, expression, and purification of recombinant ERp57 and deletion mutants. A plasmid containing the full-length cDNA of human ERp57 (pSVL-ERp57) was provided by Dr. Bourdi (NIH Bethesda, MD) [2] and utilized to produce a recombinant protein expressed in *E. coli* as fusion protein to facilitate its purification. Coding sequence of mature protein, devoid of N-terminal presequence (residues 1–24), was amplified by means of PCR using synthetic primers containing specific restriction sites, *Bam*HI and *Eco*RI, respectively, in the forward and reverse primers (Fig. 1C). Amplified DNA fragment was digested with *Bam*HI and *Eco*RI and then ligated into *Bam*HI and *Eco*RI sites of pGEX-2T vector (Amersham Pharmacia Biotech) containing the glutathione transferase protein (GST) as fusion tag. Cloning was performed in JM109 *E. coli* by standard procedures [20] while protein was expressed in BL21 *E. coli*. Cells were grown at 30 °C with shaking until the A_{600} reached 0.6 OD and then induced with 1 mM IPTG at 30 °C for 3 h. Cells were harvested by centrifugation and resuspended in NEN buffer (20 mM Tris–HCl, pH 8.0, 100 mM NaCl, and 0.5 mM EDTA) containing 0.5% Triton X-100 and 0.5 mM PMSF. Cell suspension was lysed by sonication and cleared by centrifugation at 10,000*g* for 10 min. Supernatant was passed three times over a glutathione–Sepharose column (Amersham Pharmacia Biotech), washed with 5 volumes of NEN buffer containing Triton X-100, and then with 20 volumes of NEN buffer. Fusion protein was eluted with 20 mM glutathione, 100 mM Tris–HCl, pH 8.0, and 0.5 mM EDTA and dialyzed against 20 mM Tris–HCl, pH 8.0, and 20 mM NaCl. Protein was subjected to a further purification and applied on a Macro-Prep Q column (BioRad) eluted with 10 volumes of a linear 20–250 mM NaCl gradient in 20 mM Tris–HCl, pH 8.0.

Several mutant forms of recombinant ERp57, devoid of selected domains, were obtained by PCR utilizing the pSVL-ERp57 plasmid and expressed as fusion proteins with pGEX-2T vector (Fig. 1A). To remove the external domains (a and a'), selected forward and reverse primers, respectively, containing *Bam*HI and *Eco*RI restriction sites and flanking the coding region to be amplified, were employed (Fig. 1C). Amplified fragments were subjected to *Bam*HI and *Eco*RI digestion and then ligated into pGEX-2T. Removal of internal domains (b and b') was achieved by a two-step procedure (Fig. 1D). At first, two selected primers, both containing a *Bgl*II restriction site, were used to amplify a pSVL-ERp57 vector, excluding the coding region of domains to be deleted. Amplified DNA was then digested with *Bgl*II and subjected to self-ligation to obtain novel pSVL vectors. To obtain the coding sequence of deletion mutants, these vectors were subjected to a second round of amplification with the same primers used for the recombinant ERp57. Amplified fragments were finally digested with *Eco*RI and *Bam*HI and inserted into pGEX-2T. All mutants were expressed as fusion proteins and purified as described above for recombinant ERp57. As a control protein, GST was expressed by using pGEX-2T vector alone. SDS–PAGE of all purified proteins is shown in Fig. 2. All PCR were performed with a proof-reading enzyme (Expand PCR, Roche) and for a limited number of amplification cycles.

Southwestern analysis. Recombinant ERp57 was subject to limited proteolytic digestion with 40 U/mg of thrombin (Roche) for 4 h at 37 °C and resolved on SDS–PAGE in a 15% polyacrylamide gel. After being electrotransferred to nitrocellulose or PVDF membrane using semi-dry blotting performed in a Towbin's buffer (20 mM Tris–HCl,

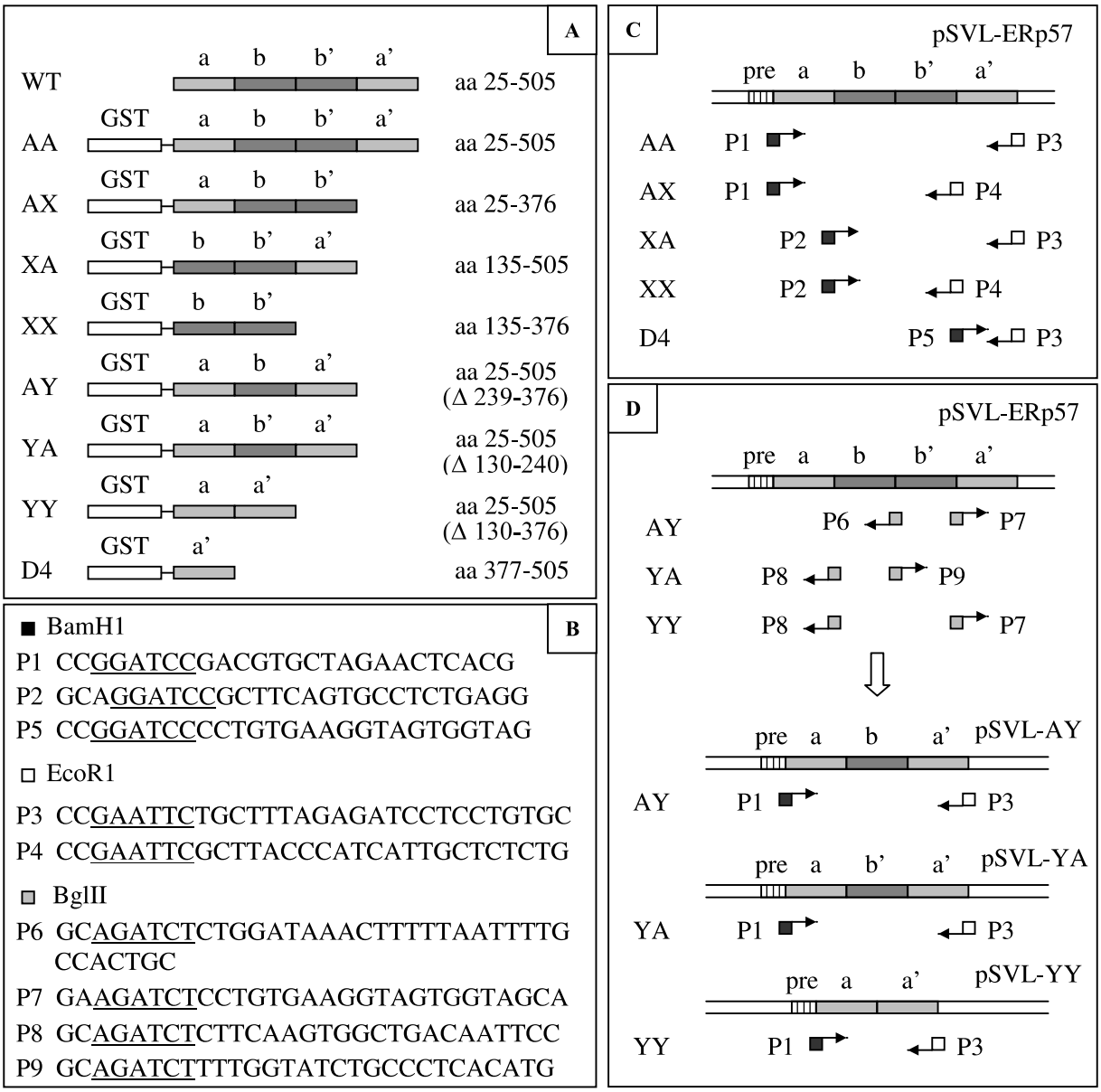

Fig. 1. (A) Schematic diagram of the domain structure of ERp57, its GST fusion protein, and several deletion mutants. Different regions of ERp57 were fused to the C-terminal end of GST, as indicated by abbreviations and the numbers of starting and ending residues. Deleted regions are given within parentheses. (B) Sequence of primers employed in PCR to obtain the recombinant proteins. Restriction sites are underlined. (C, D) Schemes of PCR employed to obtain recombinant ERp57 and all deletion mutants.

60 mM glycine, and 20% methanol), peptides were either Coomassie stained or analyzed for DNA-binding activity by Southwestern analysis [21]. For this purpose, nitrocellulose blotted peptides were denatured in 6 M guanidine HCl for 30 min at 4 °C and then renatured by serial incubation in buffer containing decreasing guanidine concentrations. The membrane was then extensively blocked in 2% BSA in TBS (12.5 mM Tris–HCl at pH 7.5, 37.5 mM NaCl, and $CaCl_2$ 10 mM). DNA-binding peptides were identified by incubation with 100 ng/ml digoxigenin labeled poly(dA)-poly(dT) (Amersham Pharmacia Biotech) in the presence of 200-fold competitor DNA from salmon sperm diluted in TBS and visualized with alkaline phosphatase-conjugated anti-digoxigenin Fab fragments (Roche). Labeling with digoxigenin was performed by nick translation, using a commercial kit (Roche). Selected peptides were excised from the Coomassie stained PVDF membrane and subjected to automatic Edman degradation using a Perkin–Elmer-AB476 gas-phase sequencer.

Results

EMSA analysis of pig liver ERp57

The aim of this work was to investigate the interactions of the protein ERp57 with DNA. ERp57 was purified from pig liver and its DNA-binding properties were analyzed by electrophoretic mobility shift assay

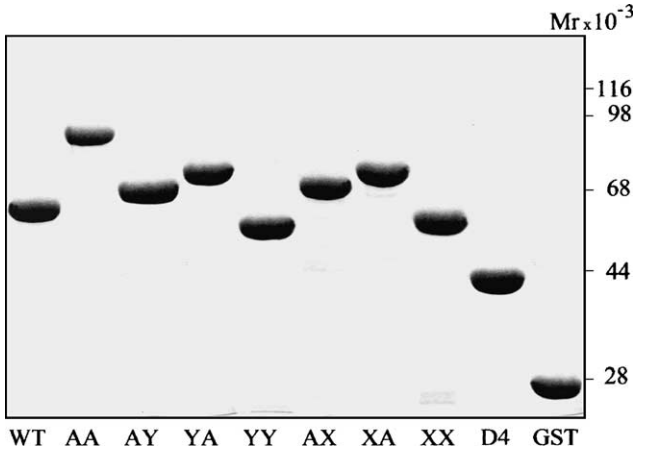

Fig. 2. SDS–PAGE analysis of purified pig liver ERp57 (WT), recombinant ERp57 (AA), and all deletion mutants (abbreviations as indicated in Fig. 1A).

(EMSA). At present, we have no information about sequence specificity of ERp57 but our previous observations indicate a preferential binding toward AT-rich regions [18,19].

Present EMSA analysis in the presence of an AT-rich fragment confirmed our previous observation. DNA–protein complexes can be observed at a protein/DNA molar ratio greater than 500:1, indicating a low affinity binding. However, increasing protein–DNA ratio leads to the formation of high-molecular weight complexes (Fig. 3A). This observation may be explained either by a cooperative binding effect with more than one protein bound to the same DNA fragment and/or to the formation of larger aggregates due to a protein–protein

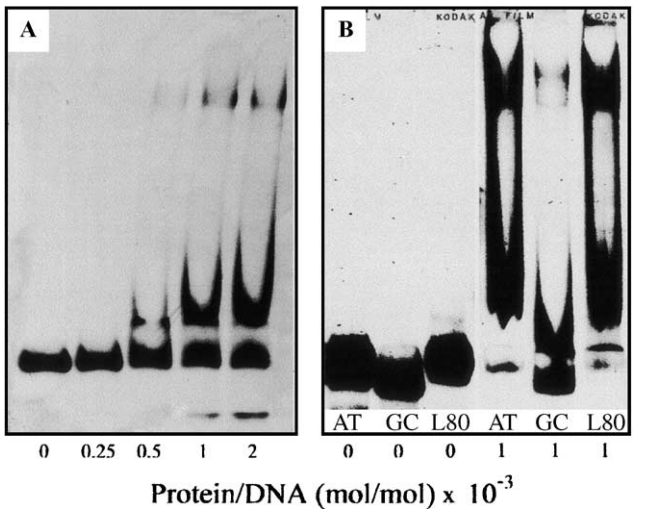

Fig. 3. (A) EMSA analysis of pig liver ERp57 with an AT-rich DNA fragment. The binding assay was performed in the presence of 1 ng labeled DNA and different amounts of protein. (B) EMSA analysis of pig liver ERp57 with 1 ng different DNA fragments varying in base composition (AT = 73%AT, GC = 33%AT, and L80 = 49%AT) and the same amount of protein.

interaction (probably induced by the DNA binding). We have observed that ERp57, when analyzed in SDS–PAGE without reducing agents, can partially exist as a dimer, indicating the protein's capability to form oligomers in solution (data not shown). EMSA analysis was also performed with a fixed amount of ERp57 and DNA fragments with similar lengths but differing in base composition. Results confirmed a preferential binding toward AT-rich regions of DNA, since a GC-rich fragment shows the lowest activity (Fig. 3B).

EMSA analysis of recombinant ERp57

To better characterize the ERp57–DNA interaction and to identify the protein domains involved in such interaction, we made use of a recombinant form of ERp57. To facilitate the purification, recombinant ERp57 has been expressed as a fusion protein with GST as an N-terminal tag domain. Moreover, the N-terminal endoplasmic localization signal present in the cloned cDNA, corresponding to aminoacids 1–24, has been removed to express the mature form of the protein.

The coding sequence of mature ERp57 has been selectively amplified from plasmid containing the full-length cDNA by means of PCR using synthetic primers containing specific restriction sites. Amplified DNA fragment was then inserted into the expression vector pGEX-2T and the resulting fusion protein GST–ERp57, here named AA, has been expressed in *E. coli* BL21 and purified by affinity chromatography on glutathione–Sepharose and ion-exchange chromatography. Following a similar procedure, we have produced several other fusion proteins devoid of selected domains of ERp57 (Figs. 1 and 2).

Recombinant GST–ERp57 was tested in EMSA and compared for its DNA-binding activity with the pig liver protein. Results show that recombinant protein behaves similarly to the wild type, with binding occurring at a slightly higher protein–DNA ratio and without the formation of high-molecular weight complexes (Fig. 4A). At present, we cannot exclude a steric hindrance of the fusion tag which weakens DNA–protein and/or protein–protein interaction. Control experiments performed with the free fusion tag showed that GST has no binding activity (Fig. 4A).

EMSA experiments have also been performed with different recombinant forms of ERp57 expressed and containing specific domain deletions. This allows us to identify and characterize the DNA-binding domain of ERp57 and elucidate any relation between the reductase activity and DNA-binding property of ERp57.

Fig. 4B shows the EMSA of all deletion mutants expressed. All proteins were analyzed in the same range of protein/DNA molar ratios. Deletion of the a' domain (mutants AX and XX) yields proteins lacking any DNA-binding activity. Conversely, deletions of the

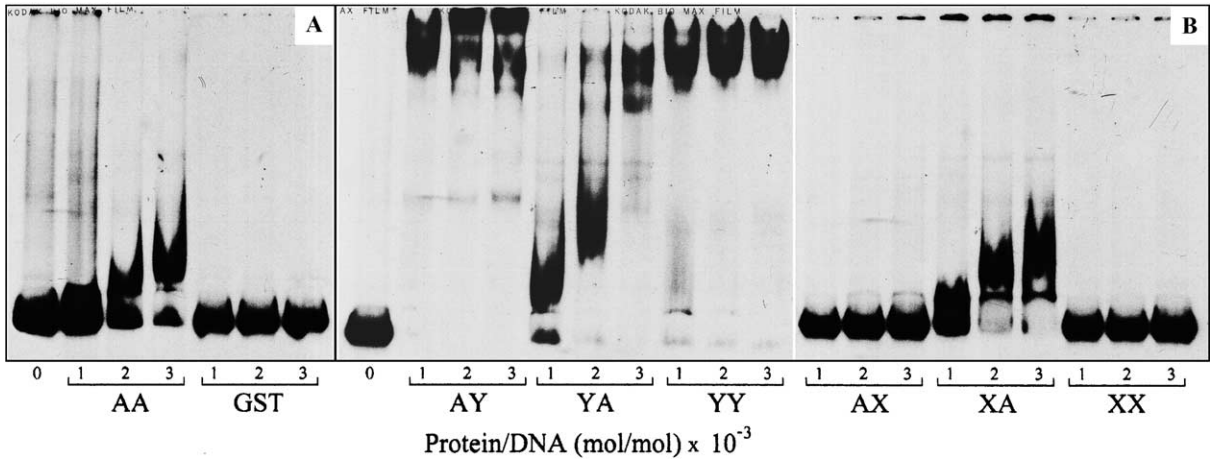

Fig. 4. (A) EMSA analysis of GST–ERp57 and GST with an AT-rich DNA fragment. (B) EMSA analysis of all deletion mutants with an AT-rich DNA fragment. The binding assay was performed in the presence of 1 ng labeled DNA and different amounts of recombinant proteins to obtain the protein/DNA molar ratio reported.

internal domains *b* and *b′* (mutants AY, YA, and YY) give rise to proteins with higher binding activity compared to GST–ERp57 (AA). In particular, the EMSA of mutants with deletion of the *b′* domain (AY and YY) shows only the presence of high-molecular weight complexes. These results strongly suggest an essential role for the *a′* domain in the interaction with DNA and lead to the conclusion that the binding site lies within this domain. Moreover, the *b′* domain can also play a role, probably through the regulation of protein–protein interaction during the formation of protein–DNA complexes. Deletions of the *b′* domain enhance the capability of mutant ERp57 to form large aggregates with DNA. This behavior seems not to be the consequence of a protein with smaller size, since it is not displayed by mutant with deletion of the *a* domain (mutant XA).

To confirm the role of the *a′* domain, we expressed a recombinant protein consisting of this domain alone, fused with GST (mutant D4), and tested its binding activity by EMSA. Fig. 5A shows this protein to be able to form complexes, even at the lowest protein/DNA ratio employed and with high affinity, thus, indicating a direct involvement of the *a′* domain in DNA binding. Considerably important is the observation that the presence of a reducing agent, such as dithiothreitol, abolishes the binding activity of mutant D4 (Fig. 5A). This is consistent with our previous data on liver ERp57 and strongly supports a specific role for the thioredoxin-like active site and the importance of protein redox state for DNA-binding activity.

Southwestern analysis of ERp57

Further evidence of *a′* domain involvement in DNA-interaction was also obtained by Southwestern analysis. GST–ERp57 was subjected to proteolytic digestion with thrombin to remove the fusion tag.

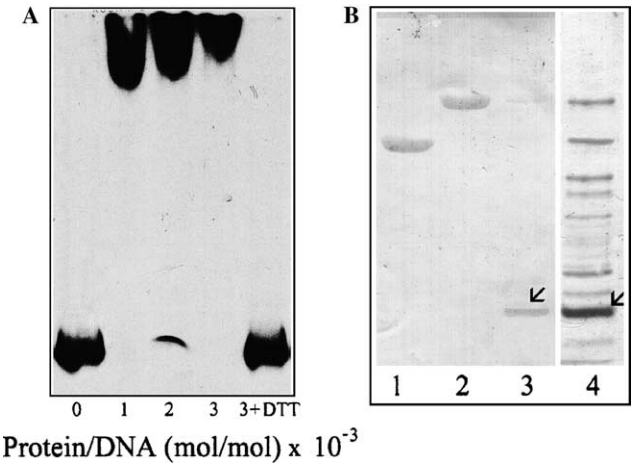

Fig. 5. (A) EMSA analysis of GST-*a′* domain (mutant D4) with an AT-rich DNA fragment. The binding assay was performed in the presence of 1 ng labeled DNA and different amounts of protein and in the presence of 10 mM dithiothreitol. (B) Southwestern analysis of pig ERp57 (lane 1), recombinant GST–ERp57 (lane 2), and peptides obtained by thrombin digestion (lane 3) resolved by SDS–PAGE. Lane 4 shows the Coomassie stained peptides. Arrows indicate the peptide subjected to Edman degradation.

Several peptides were obtained and resolved by SDS–PAGE. Fig. 5B shows that the DNA-binding activity displayed by both intact proteins, pig ERp57 and GST–ERp57, is retained in a peptide of 20 kDa. Edman degradation performed on this peptide yields the sequence TAKGEK corresponding to the C-terminal portion (330–505) of the protein and including the *a′* domain.

Discussion

It has been demonstrated that ERp57 is involved, through its reductase activity, in the correct folding and

disulfide rearrangement of misfolded proteins. However, there is growing evidence to suggest that the functional role of the protein is also related to the cellular distribution and the interactions established with other macromolecules.

The reported nuclear presence of ERp57 and its capacity to associate or react with identified nuclear components exerting a regulatory effect on several biological processes suggest that ERp57 has a role in the transcriptional activation of DNA sequences. This could take place through a redox mechanism involving nuclear proteins. A recent study has described redox processes involving either PDI or ERp57 in the control of the activity of the transcription factor E2A [22]. Otherwise, the association of ERp57 with transcription factors might modulate the binding to DNA, by possibly contributing to its intrinsic DNA-binding activity. This might be the case of the STAT3–ERp57 complexes, although they have not yet been detected in the nucleus. In this context, it should also be noted that ERp57 has been shown to modify the interaction between specific nuclear proteins and the regulatory domains of interferon-inducible genes [23].

The DNA-binding properties of ERp57 have been confirmed in this work. By means of expression of a recombinant ERp57 and several mutants containing specific domain deletions, we demonstrated that the a' domain is responsible for the binding properties. Moreover, the redox state of this domain is essential for the DNA-binding activity and the presence of reducing agents has a negative effect on such interaction. This observation is consistent with our previous results and with the identification of ERp57 as an internal nuclear matrix component (a protein fraction extracted by using the reducing agent dithiothreitol). If the binding of ERp57 to DNA is involved in transcription regulation, this will be dependent on the redox potential of the nucleus. However, contrary to what happens with many transcription factors, the interaction with DNA will be favored by more oxidizing conditions.

From our results, the b' domain might influence the DNA-binding properties of ERp57 and consequently it could be a good interaction site for other macromolecules which can regulate ERp57 activity. The identification of the ERp57 binding domains and knowledge of the proteins involved in these interactions will help elucidate the biological role of ERp57.

Acknowledgments

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