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Binding of the Protein Disulfide Isomerase Isoform ERp60 to the Nuclear Matrix-Associated Regions of DNA

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Abstract Protein ERp60, previously found in the internal nuclear matrix in chicken liver nuclei, is a member of the protein disulfide isomerase family. It binds DNA and double helical polynucleotides in vitro with a preferential recognition toward the matrix-associated regions of DNA and poly(dA)·poly(dT), and its binding is inhibited by distamycin. ERp60 can be cross-linked chemically to DNA in the intact nuclei, suggesting that its association with DNA is present in vivo. As a whole, these results indicate that ERp60 is a component of the subset of nuclear matrix proteins that are responsible for the attachment of DNA to the nuclear matrix and for the formation of DNA loops. A distinctive feature of this protein, which has two thioredoxin-like sites, is that its affinity to poly(dA)·poly(dT) is strongly dependent on its redox state. Only its oxidized form, in fact, does it bind poly(dA)·poly(dT). The hypothesis can be made that through the intervention of ERp60, the redox state of the nucleus influences the formation or the stability of some selected nuclear matrix–DNA interactions. J. Cell. Biochem. 72:528–539, 1999.

Key words: protein disulfide isomerase; chicken liver nuclei; nuclear scaffold attachment regions; DNA–protein interaction

The nuclear matrix is the insoluble skeletal structure of the eukaryotic nucleus that provides attachment sites for DNA, and hence organizes the chromatin in topologically defined looped domains. Besides its structural role, the nuclear matrix is thought to be the site of occurrence or regulation of important biological processes. DNA replication appears to be associated with the nuclear matrix and the DNA regions attached to the matrix proteinaceous meshwork (Matrix- or scaffold-associated regions (MARs or SARs), hereafter called S/MARs [Bode and Maass, 1988]), have been shown to be involved in the control of gene expression and to constitute the borders of independently regulated looped domains; the nuclear matrix is also involved in processing the pre-mRNA, being associated with the ribonucleoprotein particles and pre-mRNA splicing machinery [reviewed by Gasser and Laemmli, 1987; Berezney, 1991; Van Driel et al., 1991].

Taking into account these important structural and functional aspects of nuclear matrix–nucleic acid interaction, the study of the protein–DNA complexes at the matrix level has attracted wide interest. DNA fragments corresponding to S/MARs have been characterized in numerous instances; a systematic cloning and sequencing of these regions was recently conducted [Boulikas, 1995]. Although the S/MARs are now recognized as having a great variety of base composition and sequences, in many cases they contain A/T-rich regions, are often characterized by ATATTT motifs and topoisomerase II cleavage consensus sequences [Mirkovitch et al., 1984; Gasser and Laemmli, 1986; Cockerill and Garrard, 1986], and sometimes have high potential for unwinding [Bode et al., 1992]. Therefore, we now have a fairly good knowledge of the DNA moiety of the matrix–nucleic acid complexes, or at least of its more common features.

Much less satisfactory is the situation with regard to the protein moiety of the complexes. This is attributable to various reasons. First, the protein composition of the nuclear matrix is

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very complex, and this makes the identification of the proteins responsible for binding the DNA a difficult task. Furthermore, it appears that some DNA–protein interactions at the matrix level require an aggregate of proteins, rather than a single protein species [Ludérus et al., 1994; Zhao et al., 1996; Ferraro et al., 1996]. However, there are cases in which a particular protein has been found to interact with S/MAR sequences, sometimes with high affinity. Proteins belonging to this class are the matrins [Hakes and Berezney, 1991], ARBP [von Kries et al., 1991], SATB1 [Dickinson et al., 1992], ACBP [Hofmann and Gasser, 1991], SP120 [Tsutsui et al., 1993; von Kries et al., 1994], SAF-A [Romig et al., 1992], SAF-B [Renz and Fackelmayer, 1996; Oesterreich et al., 1997], nucleolin [Dickinson and Kohwi-Shigematsu, 1995], histone H1 [Izaurralde et al., 1989], and topoisomerase II [Adachi et al., 1989]. Although some of these proteins recognize with great affinity a consensus sequence, none has a very stringent sequence specificity, as they are able to bind a variety of S/MARs from different genes and organisms. In some cases, transcription factors, with their stringent specificity of sequence binding, are found as components of the nuclear matrix. This is the case, for example, with Sp1, ATF, OCT-1, C/EBP, CCAAT, and AP-1 [Stein et al., 1991; van Wijnen et al., 1993]. However, this remains a partial picture of the subset of DNA-binding proteins of the nuclear matrix, so that a more detailed knowledge of this class of proteins is needed to fully understand both the function and mode of action of the nuclear matrix.

In order to overcome some of the problems encountered in the search for these proteins, we resorted to exploiting a DNA–protein cross-linkage procedure whereby DNA–protein complexes present in the intact nucleus can be stabilized and subsequently characterized, thus avoiding the possible alterations in DNA–protein interactions that might occur upon disruption of nuclei. Heavy metals have been shown to induce the formation of stable cross-linkages between DNA and nonhistone proteins in intact cells, and proteins of the nuclear matrix have been found in such complexes [Wedrychowski et al., 1986]. We were further able to show that the main protein components of the cross-linkages induced by *cis*-diammine dichloro platinum (*cis*-DDP) in chicken liver nuclei were, in fact, nuclear matrix proteins [Ferraro et al., 1992]. After these observations, we began a screening of these DNA-cross-linked proteins with antibodies against known matrix proteins.

In this report, we describe how this approach led to the finding that the protein ERp60 is present in this subset of proteins from chicken liver nuclei. ERp60, also known as ERp57 [Hirano et al., 1995], Erp61, or GRP58 [Mazzarella et al., 1994], was originally misidentified as an isoform of phospholipase C [Bennett et al., 1988]. ERp60 is, instead, a member of the large protein disulfide isomerase family [Freedman et al., 1994], found originally in the endoplasmic reticulum. A variety of functions has been attributed to this protein in the past. Although the presence of two thioredoxin-like active sites leaves little doubt that its function is related to the redox properties of these sites, the precise biological role of this protein is uncertain.

A nuclear localization of ERp60 might have been suggested for the first time by gel shift experiments showing that this protein alters complex formation between nuclear proteins and the regulatory domain of interferon-inducible genes [Johnson et al., 1992]. We subsequently identified ERp60 as a component of the internal nuclear matrix in chicken cells [Altieri et al., 1993]. A nuclear localization of this protein was also described in rat spermatids and spermatozoa [Ohtani et al., 1993].

We are now able to demonstrate that, in addition to interacting with DNA in the nucleus, as shown by cross-linking experiments, ERp60 can recognize the base sequences characteristic of S/MARs. These findings strongly support the view that the ERp60 present in the nucleus is another member of the group of nuclear matrix proteins responsible for the anchorage of DNA by the nuclear matrix. Furthermore, an interesting feature of the ERp60–DNA interaction is its dependence on the redox state of the protein. This suggest that the two thioredoxin-like sites have a new, as yet unrecognized regulatory function, rather than a catalytic one.

MATERIALS AND METHODS

Purification of ERp60

ERp60 was purified from the internal matrix of chicken liver nuclei as described [Altieri et al., 1993], omitting the final dialysis step. The protein was further purified by chromatography on a hydroxyapatite column (CHT-II, Bio-Rad, Richmond, CA), from which it was eluted

with a linear gradient (0–500 mM) of K-phosphate buffer, pH 7.2.

Cross-linking Reaction

The cross-linking on intact nuclei from chicken liver by means of *cis*-DDP (Sigma Chemical Co., St. Louis, MO) and the isolation of the proteins from the cross-linked DNA–proteins complexes by means of hydroxyapatite (BioRad) were performed as described previously [Ferraro et al., 1992].

The DNA from the cross-linked complexes was isolated by gel filtration on a Sephacryl HR 400 column (Pharmacia, Uppsala, Sweden), followed by filtration on nitrocellulose membrane and dissociation of DNA from the complexes with thiourea [Ferraro et al., 1996].

Southwestern Assay

For the Southwestern blotting procedure [Bowen et al., 1980], after electrotransfer on nitrocellulose membrane, the proteins were renatured in solutions of decreasing guanidine concentration [Du Bois et al., 1990]. DNA isolated from the cross-linked complexes or poly(dA)·poly(dT) were used as probes, both labeled with digoxigenin (DIG), in the presence or absence of competitor DNA from *Escherichia coli* or salmon sperm.The membrane was then washed and stained [Mühlegger et al., 1990]. Labeling with DIG was performed by nick-translation, using the kit from Boehringer Mannheim (Germany).

Filter Binding Assay

Poly(dA)·poly(dT) was labeled by nick-translation (Boehringer Mannheim) with [^{33}P]-dATP and incubated with different amounts of purified Erp60. The reaction was performed in a final volume of 750 µl of incubation buffer (10 mM Tris-HCl pH 8.0, 80 mM NaCl) plus 0.05 mg/ml of bovine serum albumin (BSA), for 10 min at 30°C. Samples were filtered through GF/C glass microfiber filters (Whatman) prewetted in incubation buffer for 30 min. The filters were washed twice with 1.5 ml of incubation buffer. The amount of filter-bound [^{33}P]-poly(dA)·poly(dT) was determined by liquid scintillation counting.

Dot-Blot Assay

Dot-blot assay was performed on nitrocellulose membrane at constant ERp60 concentration with varying different competitor concentrations. In a typical experiment, 75 ng of ERp60 in 12.5 mM Tris-HCl, pH 7.5, and 37.5 mM NaCl (TBS Buffer) were applied to the membrane. After sample application, the membrane was incubated for 120 min at room temperature in TBS plus 2% BSA and then washed three times for 10 min each in TBS. The membrane was overlaid with 1 ml of solution containing 100 ng of different DIG-labeled DNA or poly(dA)·poly(dT) as a probe in the presence of increasing amount of competitors, as indicated in the figure legends. The competitors used were poly(dA-dT), poly(dG-dC), poly(dG)·poly(dC) (all from Pharmacia), and supercoiled DNA from the plasmid ϕX174 (New England Biolabs, Beverly, MA). The membrane was then washed and stained [Mühlegger et al., 1990]. The quantitative evaluation of the binding was performed with an Image Master system (Pharmacia).

Avidin-Biotin Complex DNA-Binding (ABCD) Assay

The effect of distamycin on ERp60-poly(dA)·poly(dT) interaction was tested by a modification of the ABCD method [Glass et al., 1987]. An excess of poly(dA)·poly(dT), biotinylated by reaction with photobiotin [Forster et al., 1985], was added to a suspension of immobilized streptavidin (UltraLink Plus; Pierce, Rockford, IL) in 20 mM Na-phosphate buffer, 0.5 M NaCl, pH 7.5. After washing with the same buffer, aliquots of this suspension corresponding to 1 nmol of streptavidin were centrifuged and the precipitates suspended again in 50 µl of buffer (12.5 mM Tris-HCl, 37.5 mM NaCl, 0.1% BSA, pH 7.5) containing 0.5 µg of ERp60, with or without 50 mM distamycin. After 1 h at room temperature, the suspensions were centrifuged and aliquots of the supernatants containing the protein remaining unbound to the immobilized poly(dA)·poly(dT) were spotted on nitrocellulose membrane. The latter was then stained by Western blotting and analyzed by scanning densitometry. To evaluate the contribution of unspecific adsorption of the protein to the immobilized streptavidin matrix, control tests were performed in the same way but using immobilized streptavidin untreated with the biotinylated poly(dA)·poly(dT).

Other Procedures

Gel permeation chromatography of native, purified ERp60 was performed on a Biosep-

S2000 column (Phenomenex, Torrance, CA), equilibrated with 0.1 M NaCl in 20 mM HEPES buffer, pH 7.5, and standardised with bovine serum IgG, BSA, ovalbumin, and chicken annexin V.

Proteins were analyzed by monodimensional sodium dodecyl sulfate (SDS) gel electrophoresis in 10% polyacrylamide, and by two-dimensional electrophoresis using for the first dimension either Ampholine (Pharmacia) [O'Farrell, 1985] or Immobiline strips (Pharmacia) [Görg, 1994]; the gels were stained with Coomassie Blue. For Western blotting, a polyclonal antibody [Altieri et al., 1993] was used, followed by biotin-conjugated anti-rabbit IgG and an avidin-biotinylated alkaline phosphatase complex (Pierce). Total S/MAR fragments from chicken liver nuclei were isolated by the LIS (3,5-diiodosalicylic acid, lithium salt) method [Mirkovitch et al., 1984]. The *Drosophila* S/MAR from the histone gene region [Mirkovitch et al., 1984] was kindly provided by Dr. Elena Mattia. S/MARs were labeled with DIG by nick-translation (Boehringer Mannheim).

RESULTS

Properties of Nuclear ERp60

Native ERp60, purified from the internal nuclear matrix of chicken liver nuclei, was run on an immobilized pH gradient, and showed an isoelectric point of 5.6 (Fig. 1B). This value is close to those calculated from the amino acid compositions of known ERp60s (e.g., 6.23 for the bovine enzyme or 5.88 for the rat enzyme). In two-dimensional denaturing electrophoresis, ERp60 showed the expected molecular mass value of 57 kDa and an apparent isoelectric point of 5.8–6.1 (Fig. 1A). From the intensity of the spot corresponding to ERp60, the amount of the protein could be evaluated as being in the order of 1% of the total protein content of the internal nuclear matrix.

Native ERp60 was also analyzed by gel permeation and its elution volume corresponded to an apparent, anomalous molecular mass of 40 kDa (data not shown), suggesting a very compact and symmetrical shape, and ruling out an aggregation of its subunit into a polymeric structure, at least in the experimental conditions used.

Cross-linking Experiments

The proteins from the nuclear matrix of chicken liver have been previously analyzed by two-dimensional electrophoresis, and those bound or close to DNA have been detected by means of a cross-linking reaction performed on intact nuclei [Ferraro et al., 1992]. In order to check the proximity of ERp60 to DNA, nuclei purified from adult chicken liver were treated with *cis*-DDP under conditions known to induce cross-linkages between proteins and DNA. The proteins cross-linked to DNA were isolated [Ferraro et al., 1992], subjected to SDS-gel electrophoresis, and analyzed by Western blotting using a polyclonal antibody elicited against ERp60 [Altieri et al., 1993]. As shown in Figure 2, the protein was present among the cross-linked nuclear components. However, also another iso-

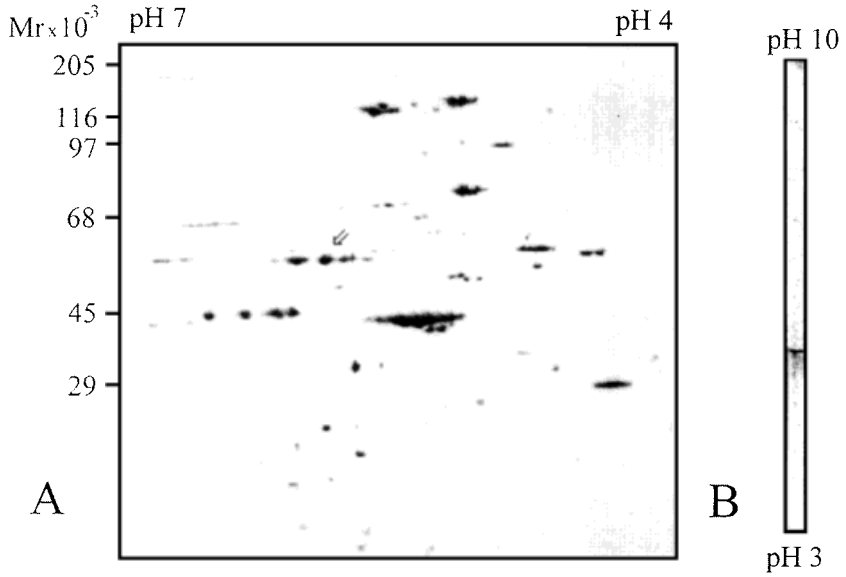

Fig. 1. **A:** Two-dimensional electrophoresis of the proteins from the internal nuclear matrix of chicken liver nuclei. Arrow, ERp60, identified by elution and partial sequencing [Altieri et al., 1993]. **B:** Electrophoresis of native purified ERp60 on immobilized pH gradient (Immobiline, Pharmacia).

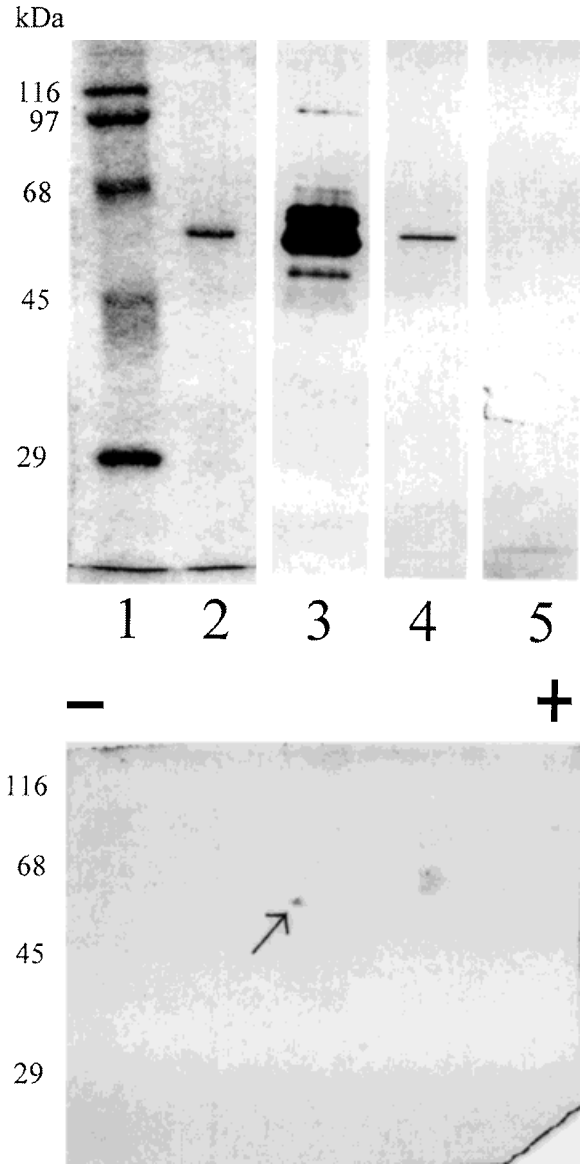

Fig. 2. Detection of ERp60 among the proteins isolated from the cross-linked complexes from chicken liver nuclei. Top: **lane 1,** molecular-weight standards. **Lane 2,** purified ERp60, Coomassie Blue-stained; **lane 3,** Western blot of purified ERp60; **lane 4,** Western blot of proteins from cross-linked complexes; **lane 5,** same as lane 2, but anti-ERp60 antibody was substituted by preimmune serum. Bottom: Western blot of a two-dimensional separation of the proteins from cross-linked complexes. Arrow, ERp60.

form of the protein disulfide isomerases, denominated PDI [Edman et al., 1985] has been detected in the nucleus, and it appeared to be localized, like ERp60, in the internal nuclear matrix (F. Altieri, unpublished results). PDI has the same M_r as ERp60, i.e., 57,000, and has a certain degree of homology with ERp60 [Freedman et al., 1994]. Therefore, in order to rule out that the Western blot results originated from a cross-reactivity of the antibody toward PDI, the Western blotting was also performed on two-dimensional electrophoresis (Fig. 2). The protein recognized by the antibody migrated as the authentic ERp60.

Binding of ERp60 to DNA

The capacity of ERp60 to bind DNA in vitro was first tested by Southwestern experiments, in which the protein, after migration in SDS-gel electrophoresis, transfer on membrane and renaturation, was treated with a labeled probe, in the presence or absence of competitor DNA from *E. coli*. Although the availability of the pure protein allowed, in principle, avoidance of the electrophoretic run, this was nevertheless carried out to make sure that any observed binding of DNA was really due to the 57-kDa ERp60, rather than to some contaminant of the protein preparation. As a probe, the DNA isolated from the cross-linked complexes was used [Ferraro et al., 1996], representing about 10% of the total genomic chicken DNA. The protein did bind DNA, and the binding was decreased, but not abolished, by an excess of competitor DNA from *E. coli* (data not shown).

The double-helical polynucleotide poly(dA)·poly(dT) is considered representative of some S/MAR regions. Therefore, it was labeled and tested for binding by ERp60 with the same type of Southwestern experiments. An efficient binding took place, as shown in Figure 3. Neither double-stranded nor single-stranded DNA from *E. coli* added in excess as a competitor affected the binding. Furthermore, after heat-denaturation of the poly(dA)·poly(dT) probe no binding was observed (Fig. 3, lane 4). This indicates that ERp60 recognizes only double-stranded polynucleotides.

Filter Binding Assay

The availability of purified ERp60 permitted study of the interaction of the native protein with DNA. An attempt to perform gel shift experiments using sonicated double-stranded poly(dA)·polydT) failed, for reasons that will be discussed later. Therefore, the interaction of ERp60 with poly(dA)·poly(dT) was studied by means of a filter binding assay, in which a fixed amount of [^{33}P]-labeled poly(dA)·poly(dT), sheared to an average length of 800 base pairs (bp), was treated with increasing amounts of protein, and the bound polynucleotide was de-

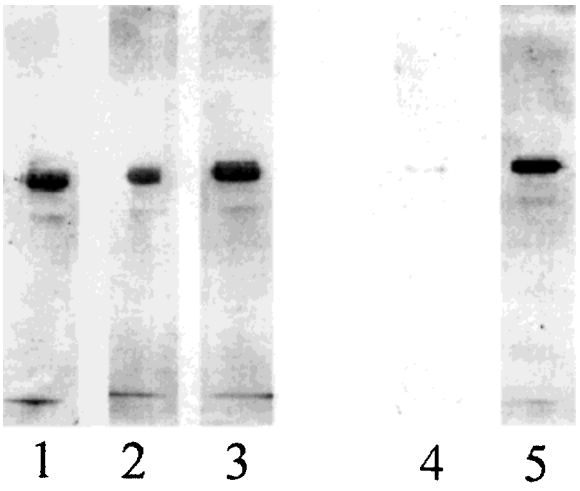

Fig. 3. Southwestern blotting of ERp60, probed with DIG-labeled poly(dA)·poly(dT). **Lane 1,** without competitor DNA; **lane 2,** with 200-fold excess *Escherichia coli* ss-DNA; **lane 3,** with 200-fold excess *E. coli* ds-DNA; **lanes 4 and 5,** 50-fold excess salmon ds-DNA; lane 4, the poly(dA)·poly(dT) probe was heat denatured.

termined by measuring the radioactivity remaining on a GF/C filter. As shown in Figure 4, the saturation curve showed no sign of cooperativity. Because of the small available amounts of purified protein, only the first part of the saturation curve could be measured, so that the results were affected by a rather large error. However, the value of K_d appeared to be in the order of 10^{-7} M. The filter binding assay was also used to study the effects of reducing or oxidizing agents on the protein, as described below.

Dot-Blot Assay

The binding specificity of ERp60 was determined by overlaying a nitrocellulose membrane, on which the native protein was spotted, with solutions of a labeled probe in the presence of various competitors. This method, although not providing a rigorous quantitative measure of the binding constants, makes it possible to evaluate the relative affinities of various base sequences using only a limited amount of protein.

Poly(dG)·poly(dC), poly(dG-dC) and, surprisingly, also poly(dA-dT) competed only slightly with poly(dA)·poly(dT), as indicated in Figure 5,showing a typical experiment. It is to be noted that these competitors were used as unsheared, high-molecular-weight polynucleotides. Poly(dA)·poly(dT),used as a competitor for control, was, as expected, a strong inhibitor.

The binding of a specific S/MAR, i.e. the histone gene S/MAR from *Drosophila* (6) was inhibited appreciably by total genomic DNA from chicken (Fig. 6A). The binding of total S/MAR fragments, isolated from chicken liver nuclei, although competed very strongly by poly(dA)·poly(dT), was instead inhibited very poorly by total genomic chicken DNA (Fig. 6B). This indicates that ERp60 recognizes preferentially some particular S/MAR sequence, not yet identified, present in the mixture of S/MAR sequences isolated from chicken liver nuclei.

The affinity of ERp60 for supercoiled DNA was tested by the use of the plasmid ϕX174 as a competitor of poly(dA)·poly(dT). As shown in Figure 5, the plasmid did not display any inhibitory effect.

Effect of Redox Reagents

Although ERp60 from chicken has not yet been sequenced, it can be expected to be relatively rich in cysteines like its counterparts of other species, all of which have a high degree of homology. In particular, all known ERp60 proteins have two thioredoxin-like active sites, each constituted by the sequence WCGHCK. It seemed of interest, therefore, to establish the effect of reducing or oxidizing reagents on the affinity of ERp60 for DNA. The protein was treated with dithiothreitol (DTT) or with diamide, and its binding to poly(dA)·poly(dT) was tested by dot-blot. The protein treated with diamide, which oxidizes the cysteine thiol groups [Kosower and Kosower, 1987], bound the polynucleotide as did the untreated protein prepared by the usual purification procedure, while the binding was nearly completely abolished by the treatment with DTT (Fig. 7). The same assay was carried out to detect the binding of poly(dA)·poly(dT) to histone H1, in order to verify that the use of DTT in this method did not lead to erroneous results. In this case, as expected, untreated or DTT-treated histone bound the polynucleotide equally well, (data not shown).

It seemed worthwhile to confirm this result by an independent method, and to this end the effect of reducing and oxidizing reagents was tested with the classical filter binding assay. The protein was incubated with [^{33}P]-labeled poly(dA)·poly(dT) for 1 h at 4°C in the presence or absence of 60 mM DTT or 60 mM diamide, and the amount of complexed polynucleotide was determined. The results fully confirmed

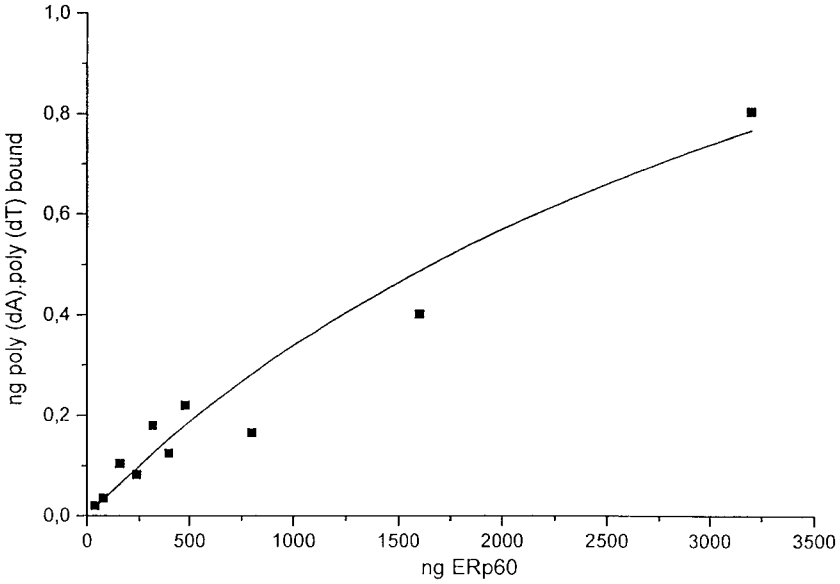

Fig. 4. Binding of poly(dA)·poly(dT) to ERp60 measured by filter binding assay. [^{33}P]-labeled poly(dA)·poly(dT) was reacted with increasing amounts of ERp60 in a final volume of 750 µl and passed through GF/C filters. The filters were washed and counted. Each point is the average of two or three measurements.

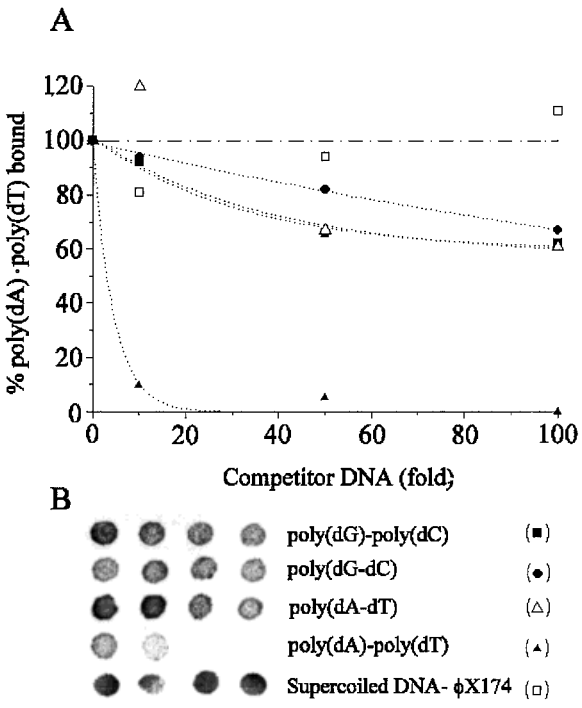

Fig. 5. Inhibition of poly(dA)·poly(dT) binding to ERp60 by different polynucleotides, measured by dot-blot assay. **A:** A total of 75 ng of protein was applied to a nitrocellulose membrane, overlaid with 100 ng/ml of DIG-labeled poly(dA)·poly(dT) in the presence of increasing amounts of poly(dA)·poly(dT) (filled triangles), poly(dA-dT) (open triangles), poly(dG)·poly(dC) (filled squares), poly(dG-dC) (filled circles), (ϕ174 plasmid (open squares). **B:** Actual stained spots, in order of increasing inhibitor concentration.

those obtained by the overlaying technique, as shown in Table I. This not only demonstrates that the state of oxidation of ERp60 is critical for its interaction with DNA, but also suggests that the most stable state of the neighboring cysteines at the active sites is the oxidized form.

Effect of Distamycin on the Binding

Since other poly(dA)·poly(dT) binding proteins interact with the minor groove of the polynucleotide, we wished to ascertain if the same holds true for ERp60. Distamycin is known to bind to the minor groove of A/T sequences, thus acting as an efficient competitor for proteins binding to the same site. However, since we found that distamycin interferes with the binding of DNA–protein complexes to nitrocellulose or to glass filters, we used a modified ABCD method [Glass et al., 1987] to investigate the effect of distamycin on ERp60-poly(dA)·poly(dT) interaction. As shown in Figure 8, distamycin effectively inhibited the binding. A similar behaviour is displayed by many other DNA-interacting proteins of the nuclear matrix [Adachi et al.,1989; Käs et al., 1989; Ludérus et al., 1994].

This result also suggests that a free minor groove is essential for the interaction of ERp60 with a double helical polynucleotide. However, although the same conclusion was reached for many other proteins whose binding is inhibited by distamycin, it should be stressed that the

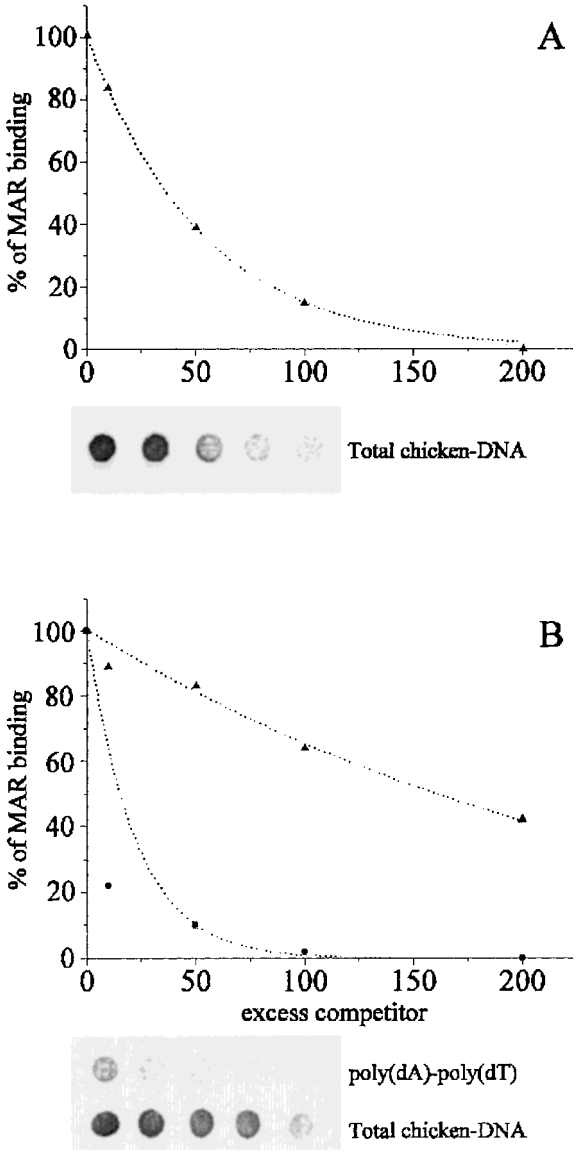

Fig. 6. Inhibition of S/MARs binding to ERp60, measured by dot-blot assay. A total of 75 ng of protein was applied to a nitrocellulose membrane, that was overlaid with (**A**) 100 ng/ml of DIG-labeled histone-gene S/MAR from *Drosophila* or (**B**) 100 ng/ml of DIG-labeled total S/MARs from chicken liver nuclei, in the presence of increasing amounts of chicken genomic DNA (filled triangles) or poly(dA)·poly(dT) (filled circles). Lower part of each graph shows the actual stained spots, in order of increasing inhibitor concentration.

drug can inhibit the binding even of proteins interacting with the major groove by causing a distortion of DNA structure [Dorn et al., 1992].

DISCUSSION

The formation of a DNA-ERp60 cross-linked complex in chicken liver nuclei shows that the protein interacts with DNA in the intact cell

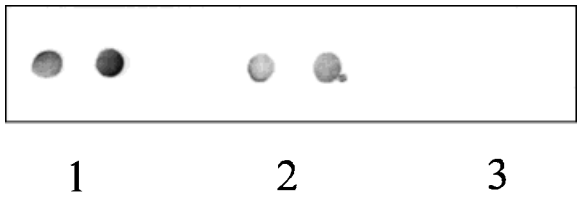

Fig. 7. Effect of redox reagents on poly(dA)·poly(dT) binding to ERp60, measured by dot-blot assay. **Lane 1,** native, untreated protein; **lane 2,** protein treated overnight at 4°C with 10 mM diamide; **lane 3,** protein treated overnight at 4°C with 10 mM DTT. A total of 75 ng of treated or untreated protein was applied in double to a nitrocellulose membrane, overlayed with 100 ng/ml of DIG-labeled poly(dA)·poly(dT).

TABLE I. Effect of Redox Reagents on the Binding of Poly(dA) · Poly(dT) to ERp60

	poly(dA) · poly(dT) bound (%)
Untreated protein	100 ± 17
Protein + diamide	99.7 ± 7.3
Protein + DTT	0.7 ± 0.5

The protein treated with diamide or DTT was reacted with either reagent at 4°C for 1 h before the addition of [^{33}P]-labeled poly(dA) · poly(dT). The mixture was then passed through GF/C filters, which were then washed and counted. The polynucleotide bound to the untreated protein was set as 100%. The averages of three or two data are shown ± the average deviations.

nucleus. Considering that ERp60 is a component of the internal nuclear matrix, this finding suggests that this protein is one of those participating in the anchorage of the DNA loops at the matrix. However, the cross-linking experiments do not allow us to ascertain whether ERp60 has by itself an affinity for DNA or rather requires to be part of a multiprotein aggregate in order to bind to the nucleic acid, as often happens with the proteins of the nuclear matrix.

The in vitro binding experiments clearly demonstrate that pure ERp60 does, indeed, bind double-stranded DNA and displays a specificity of recognition of certain base sequences. While the sequences to which the protein is actually bound in vivo remain unknown, it appears that ERp60 recognizes preferentially the S/MARs. Moreover, the protein seems to bind preferentially some particular S/MAR sequences, as can be inferred from the binding assay with the S/MAR fragment of the *Drosophila* histone gene showing a lower affinity than that displayed toward total S/MAR fragments (Fig. 6). Furthermore, the highest affinity was displayed toward the double-stranded polynucleotide poly(dA)·

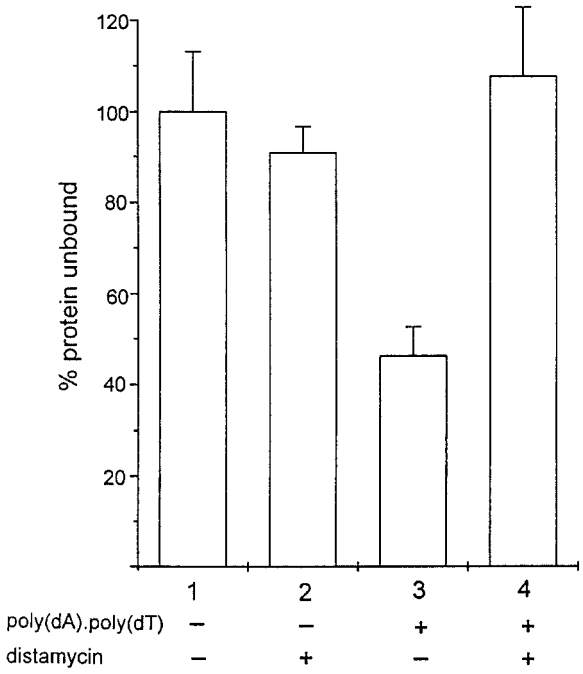

Fig. 8. Effect of distamycin on the binding of poly(dA)·poly(dT) to ERp60. The modified ABCD procedure was followed, and the ERp60 remaining unbound to the immobilized streptavidin was measured. Immobilized streptavidin was not pretreated (**lanes 1 and 2**) or was pretreated (**lanes 3 and 4**) with biotinylated poly(dA)·poly(dT). ERp60 was then added in the presence (lanes 2 and 4) or in the absence (lanes 1 and 3) of 50 mM distamycin. The amount of protein unbound to the immobilized streptavidin in control experiment 1 is set as 100%. The values indicated are the average of four measurements ±SE.

poly(dT), that is considered a good model of the A/T-rich S/MARs [Izaurralde et al., 1989] and has been found to bind well to various S/MAR-binding nuclear matrix proteins. A distinctive feature of ERp60 is its marked preference of binding poly(dA)·poly(dT) rather than poly-(dA-dT). Thus, for example, Romig et al. [1992] found that the binding of poly(dA)·poly(dT) to SAF-A is strongly dependent on the molecular weight of the polynucleotide, and that sheared poly(dA)·poly(dT), i.e., the form of polynucleotide that we use, binds to SAF-A with a much lower affinity than poly(dA-dT), exactly the opposite to what occurs with ERp60. Poly(dA)·poly(dT) is known to have particular structural features, being characterized by a narrow minor groove and, contrary to the highly flexible poly(dA-dT), has a rigid, unbendable double helix [Alexeev et al., 1987; Nelson et al., 1987]. The recognition of DNA by ERp60 might therefore be directed toward the structural features of the double helix rather than toward specific base sequences.

Some S/MAR-binding sites of the nuclear matrix recognize specifically supercoiled DNA [Tsutsui et al., 1988; Kay and Bode, 1994], and a 120-kDa protein from the nuclear matrix [Tsutsui et al., 1988] has been found to display this type of recognition. The lack of binding of the supercoiled plasmid ϕX174 in our experiments indicates that ERp60 recognizes only relaxed DNA.

Even with poly(dA)·poly(dT) the affinity (K_d in the order of 10^{-7} M) does not reach that shown by transcription factors or even S/MAR-binding proteins like ARBP [von Kries et al., 1991] for their specific base sequences (K_d on the order of 10^{-10} M). This could be explained by the fact that the base sequence recognized in vivo by ERp60, probably with higher affinity, remains unknown. The possibility should also be considered that, while this protein is capable of binding to DNA by itself, it binds with a much higher affinity when complexed with other as yet unidentified proteins. If this is the case, the sequences to which this complex is bound could be different from a typical S/MAR sequence, although it is difficult to suppose that the strong binding of ERp60 to poly(dA)·poly(dT) has nothing to do with the real interaction with DNA.

Some previous evidence [Johnson et al., 1992] supports the hypothesis of a multiprotein complex. Gel-shift experiments demonstrated that ERp60 alters complex formation between nuclear proteins and the regulatory domain of interferon-inducible genes. By the same method, no complex formation could be demonstrated between pure ERp60 and the same DNA region. Our gel-shift experiments, performed with ERp60 and poly(dA)·poly(dT), failed. This might be explained by the relatively low affinity constant for the formation of the complex. A very fast attainment of the equilibrium of the complex might also contribute to the failure of the gel shift technique.

As a whole, the results obtained indicate that ERp60 is another protein from the nuclear matrix that contributes to the anchorage of the loops of DNA. This is demonstrated by its localization in the internal nuclear matrix [Altieri et al., 1993], its interaction with DNA in intact nuclei, and its recombination in vitro with DNA, that takes place with a preferential recognition of S/MAR fragments and of S/MAR-like double-stranded polynucleotides. These features suggest that ERp60 can be classified among those

nuclear matrix proteins that bind DNA with a nonstringent specificity [Boulikas, 1995], such as histone H1, lamin B1, SP120, SAF-A and ARBP. Also these proteins bind poly(dA)·poly(dT) with high affinity. Histone H1, lamin B1 and nuclear scaffolds are sensitive, as is ERp60, to the inhibitory action of distamycin [Käs et al., 1989; Ludérus et al., 1994].

However, in contrast with the majority of these proteins, the binding of ERp60 is noncooperative, as shown by its saturation curve (Fig. 4). It should also be noted that, in contrast with SAF-A [Romig et al., 1992] and with lamins [Aebi et al., 1986], ERp60 does not appear to have a tendency to aggregate, as shown by gel filtration. Furthermore, the protein has an enzymatic activity, and precisely that of a protein disulfide isomerase [Srivastava et al., 1991; Bourdi et al., 1995; Hirano et al., 1995], that depends on the presence of thioredoxin-like active sites. All known ERp60 proteins contain two of such sites, each formed by the sequence WCGHCK. In the endoplasmic reticulum, where the ERp60 has been first identified and that is considered its usual location, it is thought to be involved in the formation and rearrangement of the disulfide bonds of the newly synthesized proteins, and in particular of N-glycosylated proteins [Elliott et al., 1997]. In its nuclear location, the role of this enzymatic activity is more uncertain. Even the nuclear location might seem surprising. However, a number of proteins of the endoplasmic reticulum have been detected inside the nucleus. This is the case, for example, of many heat shock proteins [Velazquez et al., 1980; Arrigo et al., 1980] and calreticulin [Roderick et al., 1997]. The double localization of the latter protein (which has an endoplasmic reticulum retention signal and a nuclear localization signal) has been unequivocally demonstrated [Roderick et al., 1997]. All known ERp60 proteins from vertebrates are provided with a nuclear localization signal in the proximity of their C-terminus but do not contain the usual endoplasmic reticulum retention signal KDEL, that instead is present in PDI.

Our results suggest that the two thioredoxin-like sites of the nuclear ERp60 have a role different from catalysis. In fact, the binding of ERp60 to DNA is dependent on the oxidation of at least some of its cysteines, most probably of those constituting the two active sites, since in each of these the proximity of the two cysteine residues is expected to favor the formation of the disulfide group. The dependence of DNA binding on the redox state of cysteines is well known for many proteins responsible for transcriptional regulation, such as Fos, Jun, Myb [Abate et al., 1990; Myrset et al., 1993], and many others. However, in these cases, the reduced form of cysteines is required for the binding, while for ERp60 the binding has an absolute requirement for the oxidized form.

In this regard, it should be noted that the oxidized form of ERp60 is the more stable one. This is demonstrated by the fact that in intact nuclei at least a fraction of the protein is bound to DNA, as shown by the cross-linking experiment, and by the fact that the purified protein, without the addition of redox reagents, is able to bind to DNA just like the one treated with diamide. Thus, like thioredoxin, ERp60 seems to have a lower E'_0 value than PDI, where instead the active sites are stabilized in the thiol form [Darby and Creighton, 1995].

The present data do not provide an explanation of the requirement of the oxidized form of ERp60 for binding to DNA. It is conceivable, however, that the change of redox state of the cysteines induces a conformational change in the protein, as already demonstrated in the case of Myb [Myrset et al., 1993]. A slight conformational change has also been shown to accompany the oxidation of the thiol groups at the active sites of thioredoxin [Dyson et al., 1988; Weichsel et al., 1996].

Although the redox dependence of ERp60–DNA interaction is reminiscent of that of many transcription factors, it is unlikely that ERp60 can be included in this class of proteins, not only because it is present in the nuclear matrix in relatively large amounts, that are larger anyway than those expected for the classical transcription factors, but also because its specificity of binding seems low and directed toward S/MAR-like sequences. As discussed above, nuclear ERp60 appears to be a typical S/MAR-binding protein, even if this does not rule out a possible regulatory role. In fact, the involvement of the anchorage points of DNA in the regulation of transcription, as well as in other nuclear processes, has been described extensively [Getzenberg, 1994; Bode et al., 1995; Davie, 1995; Stein et al., 1995].

ERp60 appears to represent the first case of a S/MAR-binding protein of the nuclear matrix for which the DNA-binding properties are modu-

lated by its redox state. Therefore, wherever ERp60 participates in the attachment of DNA to the nuclear matrix, the redox state of the cell and/or the nucleus might intervene as one of the factors capable of modulating the stability of this interaction.

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