Binding of a 23 kD Endonuclease to the Rat Liver Nuclear Matrix

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Abstract. In a previous paper we have described a 23 kD nuclear endonuclease (p23) that was mostly found to exist in a state of association with the isolated rat hepatocyte nuclear matrix. To investigate the nature of this interaction, the nuclear matrix was prepared using different procedures and examined for the presence/absence of the enzyme by activity gel analysis. Treatment of isolated nuclei with sodium tetrathionate (NaTT), a sulfhydryl-cross-linking agent, led to the complete recovery of p23 in the nuclear matrix, whereas incubation of nuclei with dithiothreitol (DTT), a sulfhydryl-reducing agent, led to its complete solubilization and resulting absence from the nuclear matrix. Exposure of the isolated nuclear matrix to DTT in high-ionic strength buffer, a procedure that promotes the solubilization of the internal nuclear matrix, caused the nearly complete solubilization of p23. It was concluded that disulfide bonds play an essential role in the association of p23 with the nuclear matrix and that p23 is mostly localized in the nuclear matrix interior.

Key words: Nuclear matrix — Endonuclease — Disulfide bonds — Internal nuclear matrix framework

Introduction

The nuclear matrix is an elaborate, primarily proteinaceous organization that represents the structural and, to a large extent, functional foundation of the nucleus. The nuclear matrix is generally isolated by treating purified nuclei with nucleases, followed by extraction with high-ionic strength buffers and a non-ionic detergent (Berezney and Coffey 1974; Belgrader et al. 1991). It consists of a spherical, dense peripheral nuclear lamina, a comparably delicate internal ribonucleoprotein network, one or two residual nucleoli (Berezney and Coffey 1977; Kaufmann et al. 1981), and is made up of up to one thousand different proteins (Korosec et al. 1997). The complexity of its protein content is a reflection of the involvement of

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the nuclear matrix in multifarious nuclear functions which are accomplished by different proteins associated with this structure. The nuclear matrix participates in chromatin organization, DNA replication, DNA transcription and RNA processing (Mirkovitch et al. 1984; Gerdes et al. 1994; Getzenberg 1994; Razin et al. 1995; Mattern et al. 1996; Berezney and Wei 1998).

Aside from a set of common nuclear matrix proteins found in most cells, the nuclear matrix is composed of cell type- and differentiation state-specific proteins (Stuurman et al. 1990). In addition, its protein composition and morphology depend to a certain extent on the different procedures employed for its preparation (Kaufmann et al. 1981). As the internal nuclear matrix is more sensitive to disruption and subsequent extraction than the surrounding peripheral nuclear lamina, procedures for isolating a nuclear matrix with a well-preserved internal network have been developed. They take into consideration the finding that the structural integrity of the nuclear matrix depends on the preservation of interprotein disulphide bonds and integrity of the nuclear RNA (Kaufmann et al. 1981; Belgrader et al. 1991). Namely, it has been observed that treatment of isolated nuclei with the sulfhydryl-oxidizing agent sodium tetrathionate (NaTT) prior to nuclear matrix isolation results in the enrichment of the nuclear matrix with proteins, whereas treatment with a sulfhydryl reducing agent in combination with a sulfhydryl blocking agent such as iodoacetate or N-etylmleimide favours the isolation of a nuclear matrix with a very poor internal protein content and structure (Kaufmann and Shaper 1984). The involvement of RNA in the stabilization of the internal nuclear matrix is suggested by the observation that digestion of isolated nuclei with RNaseA results in a depletion of the internal nuclear matrix network (Belgrader et al. 1991). This is hardly surprising since hnRNA in association with proteins represents the scaffolding structure of the internal nuclear matrix (Mattern et al. 1996, 1999; Barboro et al. 2002).

Procedures that favour the preservation of the internal nuclear matrix network are a prerequisite for analysing nuclear matrix-associated proteins. Comparing the protein compositions of nuclear matrices isolated using different procedures can provide information about the type of interactions of a particular protein with the nuclear matrix. Using this approach it was suggested that the interaction of nuclear mitototic apparatus protein with the nuclear matrix is RNA-dependent (Barboro et al. 2002), and that the associations of CK2 protein kinase (Zhang et al. 1998), the glucocorticoid receptor (Kaufmann et al. 1986) and topoisomerase II (Kaufmann and Shaper 1991) are mediated by disulfide bonds.

Previously we have described for the first time a 23 kD, Mg^{2+}-dependent endonuclease (p23) that was primarily associated with the rat liver nuclear matrix (Grdović and Poznanović 2003). In the present paper we attempted to determine the nature of the interactions that maintain p23 nuclear matrix-associated. To that end we isolated rat liver nuclear matrices using different protocols and examined them for presence of the enzyme. We showed that RNA integrity did not mediate the association of p23 with the nuclear matrix. Instead, the formation of disulfide bonds was critical for maintaining the enzyme bound to the nuclear matrix.

Materials and Methods

Animals

Male 30-day-old albino rats of the Wistar strain were used. The animals were kept at constant temperature, humidity and dark/light intervals.

Isolation of the nuclear matrix

All buffers contained 1 mmol/l phenylmethyl sulfonyl fluoride (PMSF). Rat liver nuclei were isolated and purified by ultracentrifugation in sucrose (Kaufmann and Shaper 1984). Nuclear matrices were isolated essentially as described previously (Poznanović et al. 1996). Freshly isolated nuclei were stabilized by an incubation at 42 ℃ for 20 min. The nuclei were then either directly subjected to endogenous nuclease digestion or treated with 0.25 mol/l sucrose, 50 mmol/l Tris-HCl (pH 7.4), 5 mmol/l $MgSO_4$ containing sulfhydryl-modifying agents. Nuclei were incubated for 1 h at 4 ℃ in the presence of either 0.5 mmol/l or 2 mmol/l NaTT in order to enrich the protein content of the nuclear matrix, or in the presence of 20 mmol/l dithiothreitol (DTT) to isolate the nuclear matrix with a lower protein content. When the protein sulfhydryl groups were reduced by DTT, the employed buffers contained 1 mmol/l DTT in all of the subsequent washing and extraction steps during nuclear matrix isolation. After incubation with NaTT, the nuclei were washed twice with 0.25 mol/l sucrose, 50 mmol/l Tris-HCl (pH 7.4), 5 mmol/l $MgSO_4$. To allow endogenous nuclease digestion, the nuclei were incubated in the same buffer overnight at 4 ℃. The nuclei were subjected to consecutive extraction/centrifugation steps: twice with high-salt buffer (2 mol/l NaCl, 10 mmol/l Tris-HCl (pH 7.4), 0.2 mmol/l $MgSO_4$), and once with freshly prepared 1 % Triton X-100 in the same buffer but without NaCl. These extractions were followed by two washes with 10 mmol/l Tris-HCl (pH 7.4) and 0.2 mmol/l $MgSO_4$. The first high-salt wash was recovered and used for SDS-polyacrylamide gel electrophoresis and activity gel analysis. Nuclear matrices were resuspended in 0.5 mol/l sucrose, 10 mmol/l $MgCl_2$, 20 mmol/l Tris-HCl (pH 7.4), 1 mmol/l PMSF to which an equal volume of sterile glycerol was added, and the matrices were kept at −20 ℃. The morphological integrity of the isolated nuclear matrix structures was checked by light microscopy.

The RNA-containing nuclear matrix was isolated as described with some modifications. All buffers contained 5 U/ml of RNAguard RNase inhibitor (Amersham Biosciences), and an extra 15 U/ml of RNAguard RNase inhibitor in both the homogenization and nuclease digestion buffers. The nuclei were treated with 2 mmol/l NaTT and all of the subsequent steps were performed as described above.

Fractionation of the nuclear matrix

To obtain nuclear matrix proteins whose anchoring to the nuclear matrix is RNA-dependent, RNA-containing nuclear matrices were resuspended at a density of 2×10^8 nuclei/ml in 10 mmol/l Tris-HCl (pH 7.4), 0.2 mmol/l $MgSO_4$, 1 mmol/l

PMSF containing 250 μg/ml RNaseA (Sigma-Aldrich) and incubated for 1 h at 4 ℃. After centifugation at $10,000 \times g$ at 4 ℃, the post-digestion wash and remaining RNase-digested nuclear matrix fraction were collected and used for SDS-polyacrylamide gel electrophoresis and activity gel analysis.

Internal nuclear matrix proteins were isolated as described by Stuurman et al. (1990). NaTT-stabilized nuclear matrices were resuspended in 10 mmol/l Tris-HCl (pH 7.4), 0.2 mmol/l $MgSO_4$ and 1 mmol/l PMSF at a density of 2×10^8 nuclei/ml. An equal volume of 4 mol/l NaCl, 40 mmol/l DTT, 10 mmol/l Tris-HCl (pH 7.4), 0.2 mmol/l $MgSO_4$ and 1 mmol/l PMSF was added to the suspension and the matrices were incubated for 20 min on ice. Insoluble nuclear matrix proteins were recovered by sedimentation at $10,000 \times g$ at 4 ℃ for 15 min. The obtained supernatant was dialyzed against 10 mmol/l ammonium acetate (pH 7.4) with several changes of buffer. The internal matrix proteins were lyophylized at −70 ℃.

Nuclease activity assay

The nuclease activity assay was performed to quantify and compare the specific endonucleolytic activities of the nuclear matrix and the internal nuclear matrix proteins. The reaction mixture (200 μl) contained 33 μg salmon sperm DNA, 100 μg of nuclear matrix or internal nuclear matrix proteins, 50 mmol/l Tris-HCl (pH 7.2), 1 mmol/l DTT and 1 mmol/l $MgCl_2$. Incubation was performed at 37 ℃ for 5, 10, 15 and 20 min. The reaction was stopped by the addition of 200 μl 1.2 N $HClO_4$. After centrifugation, the absorption ($A_{260\ nm}$) of acid-soluble oligonucleotides was measured and the relative enzymatic activity expressed as the percentage of acid-soluble DNA relative to the total DNA (assumed to be 100 %). Specific endonucleolytic activity evaluation was based on the average $\Delta A_{260\ nm}$ *per* 5 min.

SDS-polyacrylamide gel electrophoresis

A Bio-Rad Mini-Protean II electrophoresis cell was used for electrophoresis. 20 μg of proteins were loaded onto a 4 % stacking/12 % separating slab gel as described by Laemmli (1970). The gels were either stained with Coomassie Brilliant Blue R-250.

Activity gel analysis

Nuclease activities in the protein fractions were detected by activity gel analysis (zymography) essentially as described (Rauch et al. 1997). 20 μg of proteins were electrophoretically separated in 10 % SDS-polyacrylamide gels containing 100 μg/ml salmon sperm DNA. After electrophoresis, the gels were washed five times with 50 mmol/l Tris-HCl (pH 7.2) containing 1 mmol/DTT at 4 ℃, and incubated in the same buffer at 4 ℃ overnight to allow the renaturation of proteins. To activate nuclease activities, the gels were incubated for 24 h at 37 ℃ in the same buffer with the addition of 1 mmol/l $MgCl_2$. The gels were then stained with ethidium bromide for 60 min. After transillumination of the gels with UV light, nuclease activities were observed as dark areas on a fluorescent background.

Western immunoblot analysis

20 μg of proteins separated by SDS-polyacrylamide gel electrophoresis were electroblotted onto a polyvinylidene difluoride membrane. Western analysis was performed using goat polyclonal anti-lamin A/C antibody (N-18, Santa Cruz Biotechnology, Inc.). Staining was performed by the chemiluminescent technique according to the manufacturer's instructions (Santa Cruz Biotechnology, Inc.).

Quantification of proteins

Protein concentrations were determined according to the method of Lowry et al. (1951).

Results

In the present paper we examined the nature of the interaction of endonuclease p23 with the nuclear matrix by isolating nuclear matrices using different protocols and examining them for the presence of the enzyme by activity gel analysis (zymography). The structural integrity of the isolated nuclear matrix, defined as a three-dimensional nuclear substructure that consists of the spherical peripheral lamina enclosing an extensive internal nuclear matrix framework, depends on the integrity of nuclear RNA and the preservation of interprotein disulphide bonds. In order to examine whether preservation of nuclear RNA integrity is a requirement for the establishment and maintenance of the interaction of p23 with the nuclear matrix, the nuclear matrix was isolated in the continual presence of the RNase inhibitor. Shown in Fig. 1A are the electrophoretic profiles of isolated nuclei (lane 1) and nuclear matrices that were isolated either using the standard procedure (lane 3) or in the presence of RNase inhibitor (lane 5), and the corresponding high-salt extracts (lanes 2 and 4). Although the employed standard procedure of nuclear matrix isolation does not include exogenous digestion of RNA, the isolated nuclear matrix is expected to be to a certain extent RNA-depleted due to high endogenous RNase activity in nuclei. The presence of p23 endonuclease activity was examined by activity gel analysis that was performed at pH 7.2 and in the presence of Mg ions, the optimal conditions for p23 activity (Grdović and Poznanović 2003). After ethidium bromide staining, dark areas revealing the p23 endonuclease activity as well as bands at histone H1 position were observed (Fig. 1B). The observed bands at histone H1 position are due to the ability of high concentrations of histones to interfere with ethidium bromide binding to DNA (Alnemri and Litwack 1989). As can be seen from activity gel analysis on Fig. 1B, the presence of RNase inhibitor did not promote an enrichment of the nuclear matrix with p23 (lanes 3 and 5). In order to ascertain whether the nuclear matrix-bound RNA serves as a molecular bridge between p23 and nuclear matrix proteins, the obtained RNA-containing nuclear matrix was extensively treated with exogenous RNaseA (Fig. 1A, lanes 6 and 7). Activity gel analysis of the post-digestion wash and the

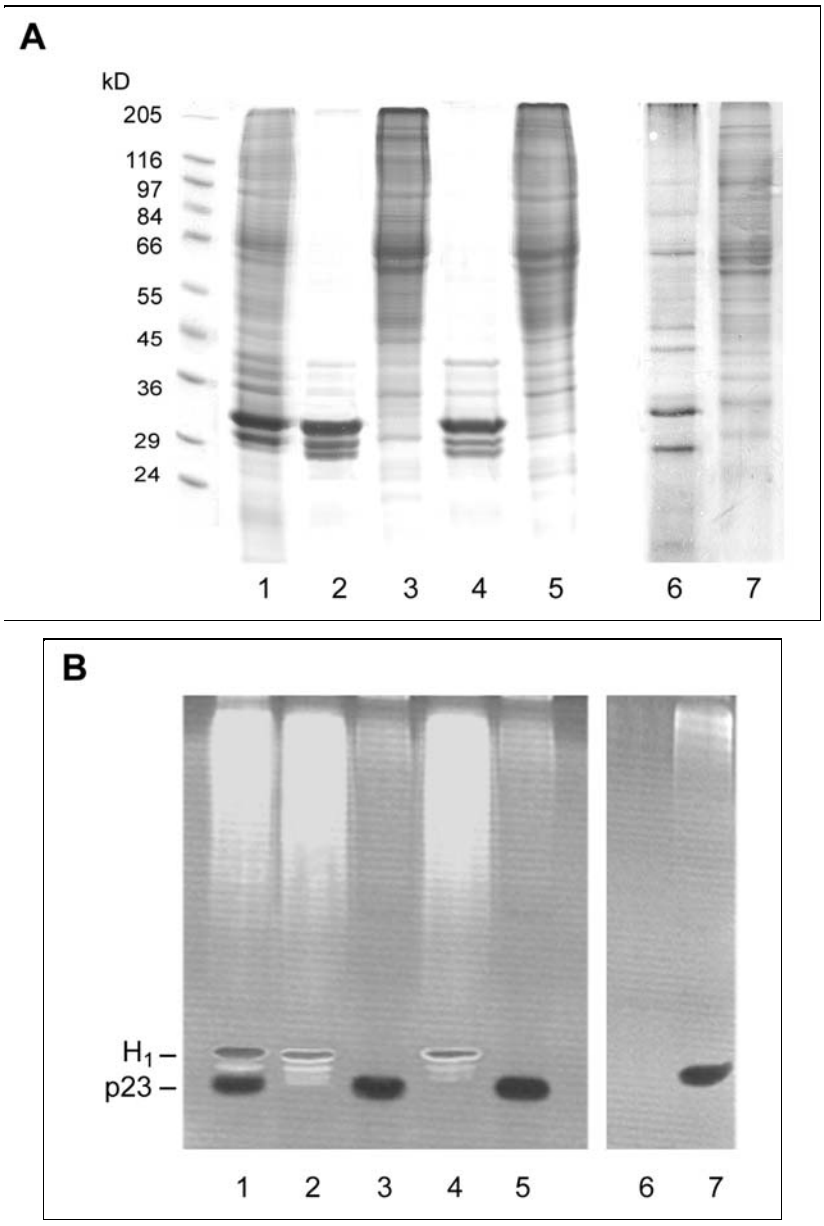

Figure 1. The effect of RNA integrity on the association of p23 endonuclease with the nuclear matrix. Coomassie staining (A) and activity gel analysis (B). Isolated nuclei, lane 1; the nuclear matrix isolated without RNase inhibitor, lane 3; the corresponding high-salt extract, lane 2. RNA-containing nuclear matrix isolated in the continuous presence of RNase inhibitor, lane 5; the corresponding high-salt extract, lane 4. Wash obtained after treatment of the RNA-containing nuclear matrix with exogenous RNaseA, lane 6; the remaining RNase-digested nuclear matrix, lane 7.

remaining RNase-digested nuclear matrix fraction revealed that p23 remained nuclear matrix-associated (Fig. 1B, lanes 6 and 7). These results suggest that the interaction of p23 with the nuclear matrix is not RNA mediated.

Next we examined the involvement of interprotein disulfide bonds on the association of p23 with the nuclear matrix. In these experiments, nuclear matrices were obtained from nuclei (Fig. 2A, lane 1) that were treated either with 0.5 mmol/l NaTT (lane 5), 2 mmol/l NaTT (lane 7), or without any treatment (lane 3). Although qualitative changes of the protein profiles were observed in the high-salt extracts with increasing concentrations of NaTT (Fig. 2A, lanes 2, 4 and 6), we could not detect any differences in the protein profiles of the corresponding isolated nuclear matrices (Fig. 2A, lanes 3, 5 and 7). Activity gel analysis revealed that treatment with NaTT affected the subnuclear distribution of p23 (Fig. 2B). Namely, when nuclear matrices were isolated in the absence of the sulfhydryl oxidizing agent, p23 was found associated with the nuclear matrix (lane 3) as well as in the high-salt extract (lane 2). Treatment with NaTT led to the complete recovery of p23 in the nuclear matrix (lanes 5 and 7) since p23 was absent from the high-salt extracts obtained after treatment of isolated nuclei with NaTT (Fig. 2B, lanes 4 and 6). These results point to a role of disulfide bonds in the association of p23 with the nuclear matrix. The detected decrese in the total amount of p23 in the fractions that were isolated without any sulfhydryl treatment (lanes 2 and 3) is the result of the different relative contribution of p23 in the high-salt and nuclear matrix fractions. Also, p23 activity in the high-salt washes is probably underestimated due to the presence of residual Na ions that inhibit it (Grdović and Poznanović 2003).

Additional confirmation of the involvement of disulfide bonds in the association of p23 with the nuclear matrix was obtained from experiments in which the nuclear matrix was isolated in the presence of the disulfide reducing agent DTT (Fig. 2A, lanes 8–11). p23 activity was examined in isolated nuclear matrices and in the extract obtained after incubation of nuclei in DTT, the post-nuclease digest wash and 2 mol/l NaCl (high-salt) extract (Fig. 2B, lanes 11, 8, 9, 10, respectively). Activity gel analysis showed that p23 was completely solubilized under conditions favouring the reduction of disulfide bonds to sulfhydryl groups. p23 activity was detected in all examined nuclear extracts released during nuclear matrix isolation (lanes 8, 9, 10), and the nuclear matrix was completely depleted of p23 activity (lane 11). These results suggest that p23 is primarily linked in the nucleus through disulfide bonds. The reduction of these bonds is the prime reason for its solubilization since p23 is extracted during nuclear matrix isolation with buffers regardless of ionic strength.

If a nuclear matrix enriched with p23 endonuclease can be isolated under conditions favouring disulfide cross-linking, we assumed that it could be possible to reverse the process and release the enzyme from this structure after disulfide reduction. In an attempt to solubilize p23 from the nuclear matrix, the procedure used to isolate the internal nuclear matrix proteins was used. It involves treatment of the NaTT-stabilized nuclear matrix with DTT in a high-ionic strength buffer

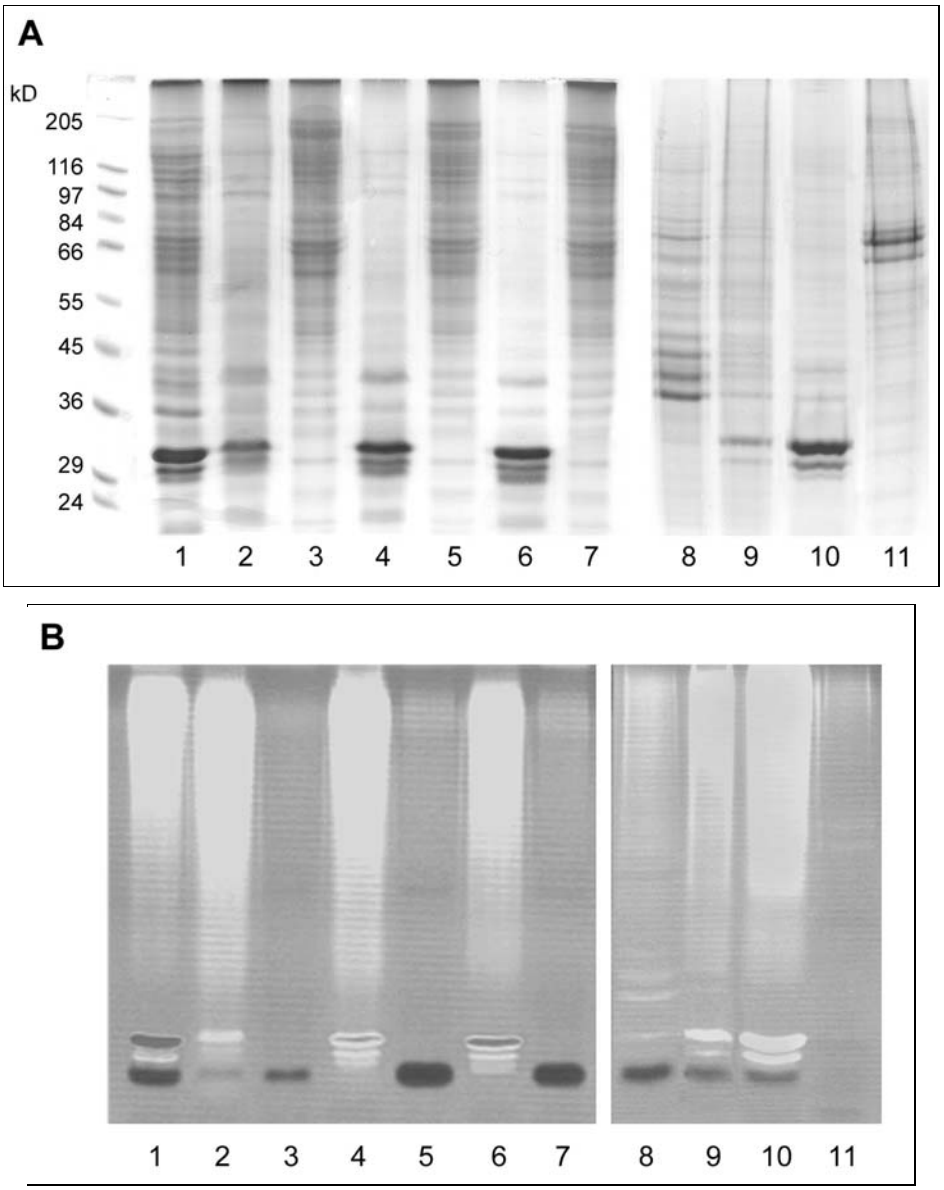

Figure 2. The effect of sulfhydryl-modifying agents on the subnuclear distribution of p23 endonuclease. Coomassie staining (A) and activity gel analysis (B). Isolated nuclei, lane 1; nuclear matrix obtained from nuclei that were not treated with any sulfhydryl-modifying agents, lane 3; the corresponding high-salt extract, lane 2. Nuclear matrices obtained from nuclei that were incubated with 0.5 mmol/l NaTT, lane 5, and 2 mmol/l NaTT, lane 7; the corresponding, respective high-salt extracts, lanes 4 and 6. Wash obtained after incubation of nuclei with 20 mmol/l DTT, lane 8; endonuclease digest wash, lane 9; high-salt extract, lane 10; the isolated nuclear matrix, lane 11.

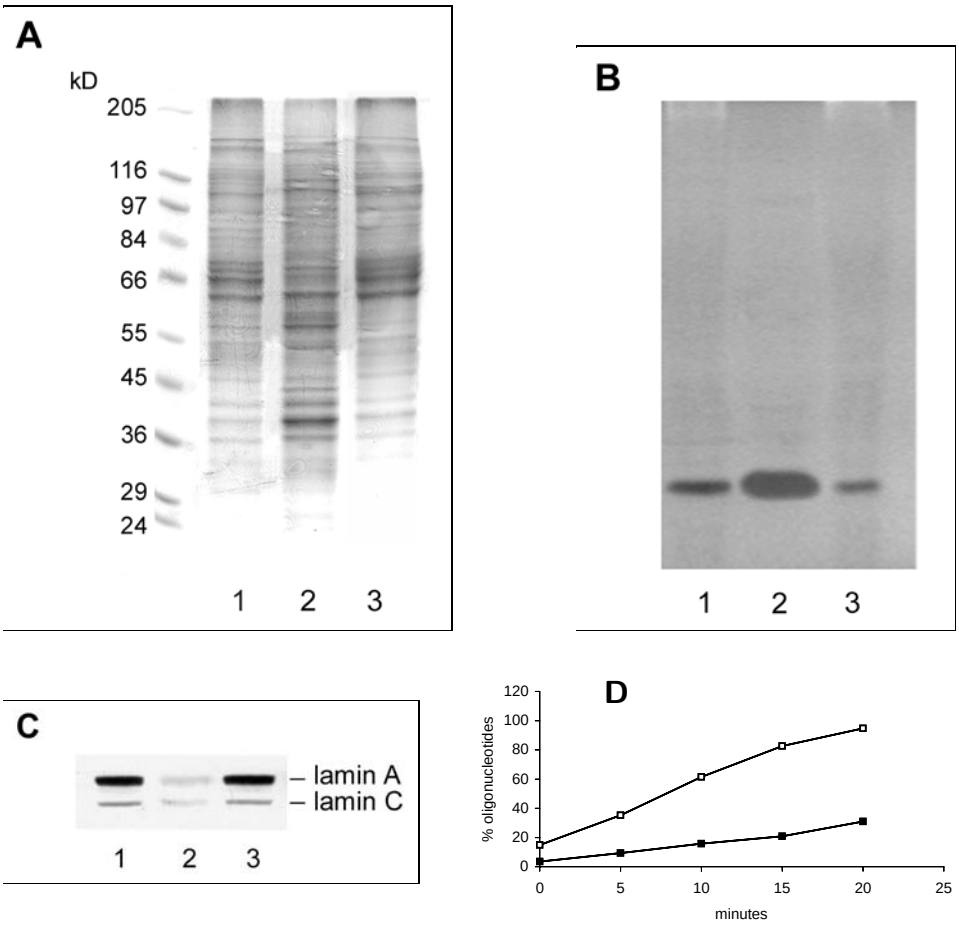

Figure 3. Solubilization of p23 endonuclease from the nuclear matrix. Coomassie staining (A) and activity gel analysis (B). The nuclear matrix (lane 1) was incubated with 20 mmol/l DTT and 2 mol/l NaCl and separated by centrifugation into the soluble internal nuclear matrix fraction (lane 2) and insoluble proteins (lane 3). C. Western immunoblot analysis with anti-lamin A/C antibody of nuclear matrix (lane 1), internal nuclear matrix (lane 2) and insoluble (lane 3) proteins. D. Nuclease activity assay of the nuclear matrix (■) and internal nuclear matrix (□) proteins. Each point was obtained from at least three separate experiments.

(2 mol/l NaCl). The obtained soluble nuclear matrix protein fraction represents the internal nuclear matrix. We established that this protein fraction consists of about 12% of the total nuclear matrix protein (electrophoretic profile in Fig. 3A, lane 2). Compared to the soluble internal nuclear matrix fraction, the insoluble fraction (electrophoretic profile in Fig. 3A, lane 3) is considerably enriched with the peripheral nuclear lamina, confirmed by Western immunoblot analysis (Fig. 3C,

lanes 2 and 3). Activity gel analysis revealed that p23 was mostly extracted from the nuclear matrix with the internal nuclear matrix fraction (Fig. 3B, lane 2); its activity in the insoluble post-extraction fraction was lower (lane 3). Since activity gel analysis cannot precisely quantify enzymatic activity, a nuclease activity assay was performed to compare the specific endonucleolytic activities of the nuclear matrix and the obtained internal nuclear matrix fraction (Fig. 3D). The assay revealed that the internal nuclear matrix had a three-fold higher specific activity than the nuclear matrix. The detected increased endonucleolytic activity was most likely the result of an increased p23 content in the internal nuclear matrix. These results led us to assume that most of the nuclear matrix-associated p23 is located in the nuclear matrix interior. Additional proof for this conclusion was obtained from experiments showing that the isolated peripheral nuclear lamina was devoid of endonucleolytic activity (results not shown).

Discussion

In the present paper we attempted to identify the type of bond/link that maintains the association of endonuclease p23 with the nuclear matrix. The importance of RNA integrity and the role of disulfides, two essential factors responsible for the preservation of the internal nuclear matrix network were examined. The persistant association of p23 with the nuclear matrix, regardless of the initial presence of either RNasin or endogenous and exogenous RNase activities suggests that RNA is not required for its interaction with the nuclear matrix (Fig. 1). Experiments with sulfhydryl-oxidizing and sulfhydryl-reducing agents suggest that the establishment of disulfide cross-links favours the partitioning of endonuclease p23 in the isolated nuclear matrix. Treatment of nuclei with the sulfhydryl-cross-linking agent NaTT lead to the complete recovery of p23 in the nuclear matrix (Fig. 2B, lanes 4–7). In contrast, when the nuclear matrix was isolated in the presence of the sulfhydryl-reducing agent DTT, p23 was completely extracted (Fig. 2B, lanes 8–11). Under intermediate conditions of disulfide/sulfhydryl oxidation/reduction, when nuclear matrices were isolated from nuclei without any sulfhydryl treatment and only exposed to oxidizing conditions favouring the spontaneous formation of disulfide bonds, p23 was divided between the nuclear matrix and high-salt extract (Fig. 2B, lanes 2 and 3). The ability of DTT to lower and NaTT to increase the amount of nuclear matrix associated-p23 suggests that its connection to the nuclear matrix involves disulfide bond formation. This was also confirmed by the capacity of DTT in a high-ionic strength buffer to solubilize p23 from the NaTT-stabilized nuclear matrix (Fig. 3B). The ease of extraction of p23 from nuclei in the presence of DTT with buffers regardless of ionic strength (Fig. 2, lanes 8–11), and the fact that solubilization of p23 from the nuclear matrix requires reduction of disulfide bonds suggests that p23 is primarily linked in the nucleus through disulfide bonds.

It is not clear if disulfide bonds between p23 and nuclear matrix proteins exist *in vivo* since during isolation nuclei are exposed to oxidizing conditions favouring

the spontaneous formation of disulfide bonds (Kaufmann and Shaper 1984). Stuurman et al. (1992) reported that most of the nuclear matrix-associated proteins that contain oxidized sulfhydryl groups in response to NaTT actually form disulfide bonds in the nuclear matrix. It seems that some specifity of NaTT-mediated interaction of nuclear matrix-associated proteins with the nuclear matrix does exists. A fusion protein composed of the nuclear localization signal of the SV-40 large T antigen and β-galactosidase was not observed in the NaTT-stabilized nuclear matrix in spite of the presence of 16 cysteine residues (Van Steensel et al. 1995). Also, a mutation of a single cysteine of the glucocorticoid receptor results in significant decrease of receptor binding in the NaTT-stabilized nuclear matrix (Van Steensel et al. 1995). Finally, it has been documented that treatment with NaTT does not change the spatial distribution of examined nuclear matrix proteins (Neri et al. 1995), arguing against the possibility of protein rearrangements. NaTT probably stabilizes native protein-protein interactions by cross-linking proximal sulfhydryl groups of neighbouring nuclear proteins (Kaufmann and Shaper 1984; Kaufmann et al. 1986).

In this paper we showed that disulfide bond formation is involved in the association of p23 with the nuclear matrix. Thus, p23 belongs to a group of nuclear proteins that becomes nuclear matrix-associated as a result of disulfide bond formation: the glucocorticoid receptor (Kaufmann et al. 1986), CK2 protein kinase (Zhang et al. 1998), SV-40 large T antigen (Humphrey and Pigiet 1987), topoisomerase II (Kaufmann and Shaper 1991) and a 62 kD protein present in metaphase chromosomes (Fields and Shaper 1988). For all of these proteins, association with the nuclear matrix was enhanced by disulfide bond formation (*in vitro* by NaTT) and diminished as a result of treatment with sulfhydryl-reducing agents. At present, the question which nuclear matrix protein(s) participate in the formation of disulfide bonds with p23 and the functional meaning of this association remain unanswered.

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