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Selected Nuclear Matrix Proteins Are Targets for Poly(ADP-Ribose)-Binding

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Abstract Recent evidence suggests that poly(ADP-ribose) may take part in DNA strand break signalling due to its ability to interact with and affect the function of specific target proteins. Using a poly(ADP-ribose) blot assay, we have found that several nuclear matrix proteins from human and murine cells bind ADP-ribose polymers with high affinity. The binding was observed regardless of the procedure used to isolate nuclear matrices, and it proved resistant to high salt concentrations. In murine lymphoma LY-cell cultures, the spontaneous appearance of radiosensitive LY-S sublines was associated with a loss of poly(ADP-ribose)-binding of several nuclear matrix proteins. Because of the importance of the nuclear matrix in DNA processing reactions, the targeting of matrix proteins could be an important aspect of DNA damage signalling via the poly ADP-ribosylation system. J. Cell. Biochem. 70:596–603. © 1998 Wiley-Liss, Inc.

Key words: poly(ADP-ribose); PARP; nuclear matrix; noncovalent interactions

Poly(ADP-ribose), a homoplymer of ADP-ribose units enzymatically derived from NAD^+, has been found in all eukaryotic cells tested so far with the exception of yeast, and it has been implicated in cellular responses to DNA damage [for reviews see Althaus and Richter, 1987; Boulikas, 1991; de Murcia and Ménissier-de Murcia, 1994; Lindahl et al., 1995]. On the basis of recent studies, poly(ADP-ribose) polymerase (PARP; E.C. 2.4.2.30) is thought to play a role downstream of DNA repair events affecting cell survival and cell death pathways. Cells from transgenic mice lacking the gene for PARP exhibit normal DNA repair functions but are nevertheless hypersensitive to γ-irradiation [Wang et al., 1997; Ménissier-de Murcia et al., 1997]. Like other DNA damage signal proteins (i.e., p53 or DNA-dependent protein kinase), PARP binds to and is activated by DNA breaks [for review see de Murcia and Ménissier-de Murcia, 1994; Lindahl et al., 1995]. Upon activation, PARP automodifies itself with multiple ADP-ribose polymers. Thus the enzyme converts into a porcupine-like structure with up to 28 polymers of different sizes and complexities extending from the protein core. These polymers may contact selected proteins in the vicinity of DNA breaks and form tight complexes with them [Althaus et al., 1998]. For example, poly(ADP-ribose) may cause the dissociation of histones from DNA [Althaus, 1992; Realini and Althaus, 1992] and block (or reverse) the sequence-specific DNA binding of p53 [Malanga et al., 1998]. In addition, polymer-protein complexes involve exceptionally strong noncovalent interactions as demonstrated by their resistance to treatment with 1 M NaCl, strong acids, detergents, and chaotropic agents [Panzeter et al., 1992a]. More recently, we have discovered a polymer-binding sequence motif of ~ 22 aa in these proteins [Althaus et al., 1998; Malanga et al., 1998]. This illustrates the specificity of polymer-protein interactions and explains why other polyanions [poly(A), tRNA] or mono-ADP-ribose and NAD^+ do not bind to these proteins [Panzeter et al., 1992; Realini et al., 1993].

The present study was undertaken to determine whether or not nuclear matrix proteins of

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human (HaCaT) and murine (L5178Y) cells are targets of poly(ADP-ribose)-binding. It is now widely accepted that DNA processing reactions are associated with the nuclear matrix. The nuclear matrix, initially identified as the insoluble structure remaining after subjecting nuclei to sequential DNase digestion and high salt extraction [Berezney and Coffey, 1974], has a fundamental role in maintaining nuclear architecture and coordinating nuclear functions [for reviews see Berezney et al., 1995; Boulikas, 1995; Stein et al., 1996]. Consistent with this role, enzymes involved in DNA replication, transcription, RNA processing, as well as regulators of these processes, have been found associated with the nuclear matrix. Moreover, a number of reports implicate the nuclear matrix as the critical target of DNA damaging agents [Bullenders et al., 1988; Fernandez and Catapano, 1995; Koehler and Hanawalt, 1996; Bouayadi et al., 1997].

Against this background, the observation that both PARP and poly(ADP-ribose) are associating with the nuclear matrix is of particular interest. In carcinogen-treated mouse fibroblasts, Cardenas-Corona et al. [1987] observed that up to 70% of endogenous poly(ADP-ribose) coisolate with the nuclear matrix, suggesting that this subnuclear structure is a major site of poly(ADP-ribose) metabolism. Alvarez-Gonzalez and Ringer [1988] have reported that the poly(ADP-ribosyl)ation system redistributes between bulk chromatin and the nuclear matrix in rat hepatocytes following partial hepatectomy. The present study demonstrates high affinity binding of ADP-ribose polymers to selected proteins of the nuclear matrix.

MATERIALS AND METHODS

Cell Cultures

Human keratinocytes (HaCaT cell line) were grown to confluence in Dulbecco's minimal essential medium supplemented with 10% foetal calf serum, as described previously [Malanga and Althaus, 1994]. L5178Y-R (LY-R) and L5178Y-S (LY-S) murine leukemic lymphoblast cell lines were maintained in suspension cultures in Fisher's medium supplemented with 8% heat-inactivated bovine serum as reported by Szumiel [1979].

Nuclei and Nuclear Matrix Isolation

All the following steps were performed at 4°C, unless otherwise indicated. PMSF (1 mM) was added to each buffer immediately before use. HaCaT cells were resuspended in 10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1% Triton X-100 and disrupted with a Polytron homogenizer. L5178Y cells were lysed in 10 mM Tris-HCl, pH 7.8, 3 mM $MgCl_2$, 1 mM EDTA, 0.5 mM dithiothreitol, Triton X-100 (0.04% for LY-R and 0.02% for LY-S cells) and homogenized with 10–12 strokes of a glass-teflon homogenizer. Following centrifugation at 800xg for 10 min, the crude nuclear pellets were resuspended in 20 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, 0.25 M sucrose and centrifuged through a layer of 0.88 M sucrose in the same buffer [Adamietz and Rudolph, 1984]. Pelletted nuclei were digested with DNase I (0.25 mg/ml) in 20 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 0.25 M sucrose, and subsequently subjected to low and high ionic strength buffer extractions (10 mM Tris-HCl, pH 7.4, 0.2 mM $MgCl_2$, in the absence or presence of 2 M NaCl) for nuclear matrix preparation, according to Berezney and Bucholtz [1981]. Where indicated in the figure legends, 2 M NaCl was substituted with 0.3 M ammonium sulphate [Cardenas-Corona et al., 1987]. The final nuclear matrix preparations were washed with and resuspended in low ionic strength buffer and stored at -20°C until use.

Since nuclear matrix preparations may retain variable amounts of chromatin proteins [Belgrader et al., 1991], we subjected them to an additional extraction with 0.4 N H_2SO_4 (cf. Fig. 3). The proteins removed by this treatment were precipitated with ice-cold TCA (20% final concentration) and included in the gel of Figure 3 as a control.

Electrophoretic Analyses

Subnuclear fractions from human and murine cells were analyzed by SDS-PAGE in a minigel system (7x9x0.1 cm), according to Laemmli [1970]. Protein samples (30 µg) were loaded onto 8–15% polyacrylamide gradient gels with 4% acrylamide as a stacking gel. Alternatively, 10% SDS-polyacrylamide gels were prepared from a stock solution available commercially (ProRanger(tm), Hydrolink, AT Biochem). Proteins were either stained with Coomassie Blue, or transferred onto nitrocellulose (0.45

μm pore size, Schleicher & Schuell, Keene, NH) using a semidry transblot apparatus (Bio-Rad).

Poly(ADP-Ribose)-Binding Assay

[^{32}P]-poly(ADP-ribose) (0.5 μCi/nmol ADP-ribose) was synthesized in vitro, as described [Panzeter et al., 1992a]. The poly(ADP-ribose)-binding assay was performed on nitrocellulose blots after removal of SDS as described earlier [Panzeter et al., 1993]. Briefly, the filters were extensively washed with a total of six changes of 10 ml TBST [10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.05% (v/v) Tween-20] in 12 hours, and then incubated with [^{32}P]-poly(ADP-ribose) [0.5 nmol of total ADP-ribose residues] in 10 ml TBST for 1 hour, at room temperature with gentle agitation. Unbound polymers were removed by extensive washes with TBST until no radioactivity could be detected in the supernatant. The filters were dried and poly(ADP-ribose)-protein complexes were visualized by autoradiography.

In previous studies it was found that polymer-protein complexes are resistant to high salt concentrations [Panzeter et al., 1992a]. Therefore, in the experiment shown in Figure 4, the blots were subjected to an additional wash with 10 ml TBST containing 1 M NaCl.

Other Procedures

Protein and DNA contents were estimated by the methods of Lowry et al. [1951] and Burton [1956], respectively.

RESULTS

Poly(ADP-Ribose)-Binding Proteins in Subnuclear Fractions From Human and Murine Cells

Nuclei were isolated from confluent human keratinocytes (HaCaT cell line) and two murine lymphoma L5178Y cell sublines (LY-R and LY-S) and subjected to sequential nuclease digestion and high salt (2M NaCl) extraction, as described in Materials and Methods. In parallel, the ammonium sulphate procedure for nuclear matrix isolation from HaCaT cells was employed. In all cases, nuclear matrices containing $<$ 4% of the nuclear DNA and 20–26% of nuclear proteins were obtained.

Salt-soluble and nuclear matrix proteins from HaCaT- and LY-cell nuclei were separated on 8%-15% SDS-polyacrylamide gradient gels (Fig. 1A,B). We observed that the two salt procedures employed for matrix isolation from HaCaT cells gave qualitatively identical results. The nuclear matrices differed only with regard to the relative amount of polypeptides in the molecular weight ranges of 50–60 KDa and 12–18 KDa (Fig. 1A).

The two closely related cell sublines, LY-R and LY-S, have been defined on the basis of their differential sensitivity to various DNA damaging agents [Alexander and Mikulski, 1961]. Such differences have been correlated with changes in the expression of nuclear proteins, possibly involved in DNA organization in loop domains [Kapiszewska et al., 1989; Malyapa et al., 1996]. Differences in the protein composition of subnuclear fractions from LY-R and LY-S cells could be observed (Fig.1B, arrows).

Proteins from duplicate gels (Fig. 1) were transferred onto nitrocellulose and analyzed in a poly(ADP-ribose)-blot assay (Fig. 2). It should be noted that the principles, assumptions, and limitations of this blot assay [Panzeter et al., 1993] are similar to those of South-Western and Far-Western blots, i.e., that SDS is removed by extensive filter washes, thereby allowing sufficient protein renaturation. Only positive signals are indicative of specific polymer-protein interactions. Therefore, histones, which bind polymers also under nondenaturing conditions in solution [Panzeter et al., 1992a,b], served as positive controls. Besides histones (and a few other proteins in the salt-soluble nuclear fractions), nuclear matrix proteins were predominant targets for poly(ADP-ribose)-binding. A comparison of the blots in Figure 2A,B also showed differences in the polymer-binding patterns between different cell types and sublines of LY-cells.

That matrix proteins were a target of poly-(ADP-ribose)-binding was confirmed by the experiment shown in Figure 3.

Nuclear matrices were subjected to acid extraction (0.4N H_2SO_4) before electrophoretic and poly(ADP-ribose)-binding analyses. This treatment was used to remove basic chromatin proteins that are found in nuclear matrix preparations in variable amount, depending on the salt conditions of extraction [Belgrader et al., 1991]. Actually, histones and/or histone-like proteins were only minor components of 2 M NaCl extracted matrices, whereas they were more abundant in matrices isolated by the ammonium

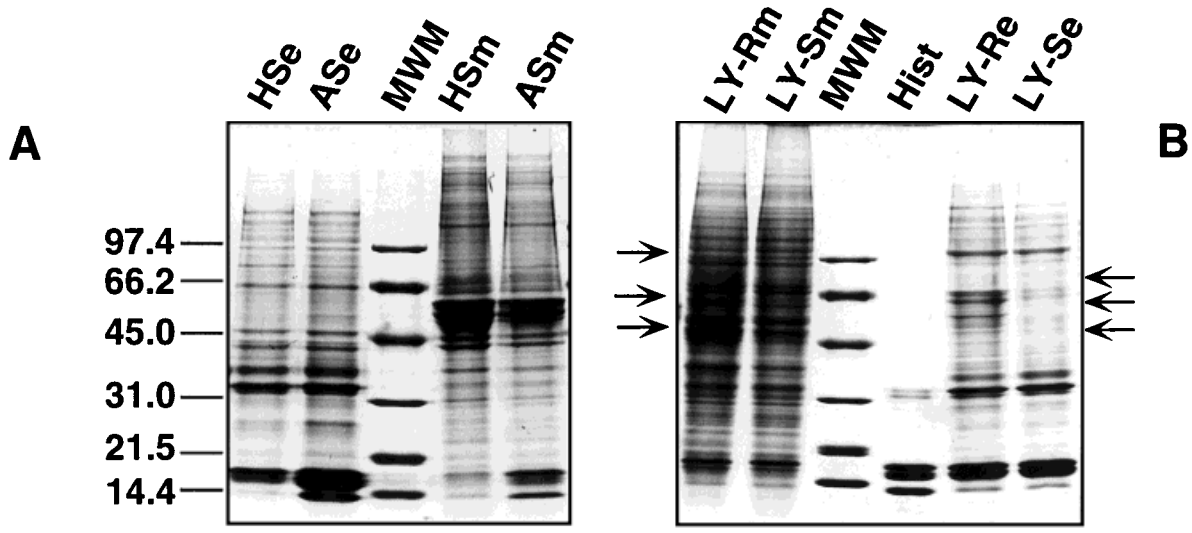

Fig. 1. Electrophoretic profiles of subnuclear fractions from HaCaT and L5178Y cells. Salt-soluble nuclear proteins (30 µg) and nuclear matrices (30 µg of protein) from HaCaT cells (**A**) and the two L5178Y cell sublines, LY-R and LY-S, (**B**) were electrophoresed on 8–15% SDS-polyacrylamide gradient gels and visualized by Coomassie Blue staining. - HSe: 2M NaCl (high salt) extract; ASe: ammonium sulphate extract; HSm: high salt matrix; ASm: ammonium sulphate matrix. LY-Rm and LY-Sm: nuclear matrices from LY-R and LY-S cells, respectively; Hist: histone H1 and core histones from calf thymus; LY-Re and LY-Se: nuclear extracts from LY-R and LY-S cells, respectively. Nuclei from LY-R and LY-S cells were extracted with 2M NaCl. Numbers on the left indicate the Mr (KDa) of molecular weight markers (MWM); the arrows point to differences in the protein composition of subnuclear fractions of LY-R and LY-S cells.

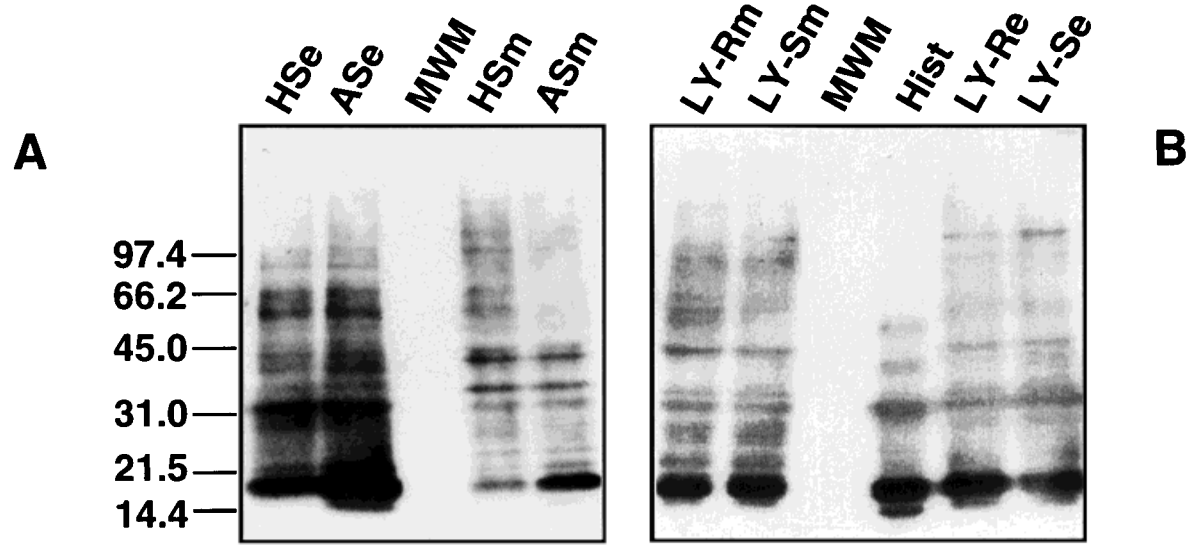

Fig. 2. Poly(ADP-ribose)-binding proteins in subnuclear fractions from HaCaT (**A**) and L5178Y (**B**) cells. Duplicates of the gels shown in Figure 1 were transferred onto nitrocellulose and probed with [^{32}P]-poly(ADP-ribose) as described in Materials and Methods. Protein-poly(ADP-ribose) complexes were visualized by autoradiography, histones (Hist) served as positive controls. Abbreviations as in Figure 1.

sulphate procedure (Fig. 3A). Importantly, most of the poly(ADP-ribose)-binding proteins identified in the nuclear matrices from both HaCaT and LY cells were not solubilized by the acid (Fig. 3B).

Salt Resistance of Poly(ADP-Ribose)-Nuclear Matrix Protein Interactions

Apart from their specificity, a hallmark of polymer-protein complexes is their resistance to high salt treatment [Panzeter et al., 1992a;

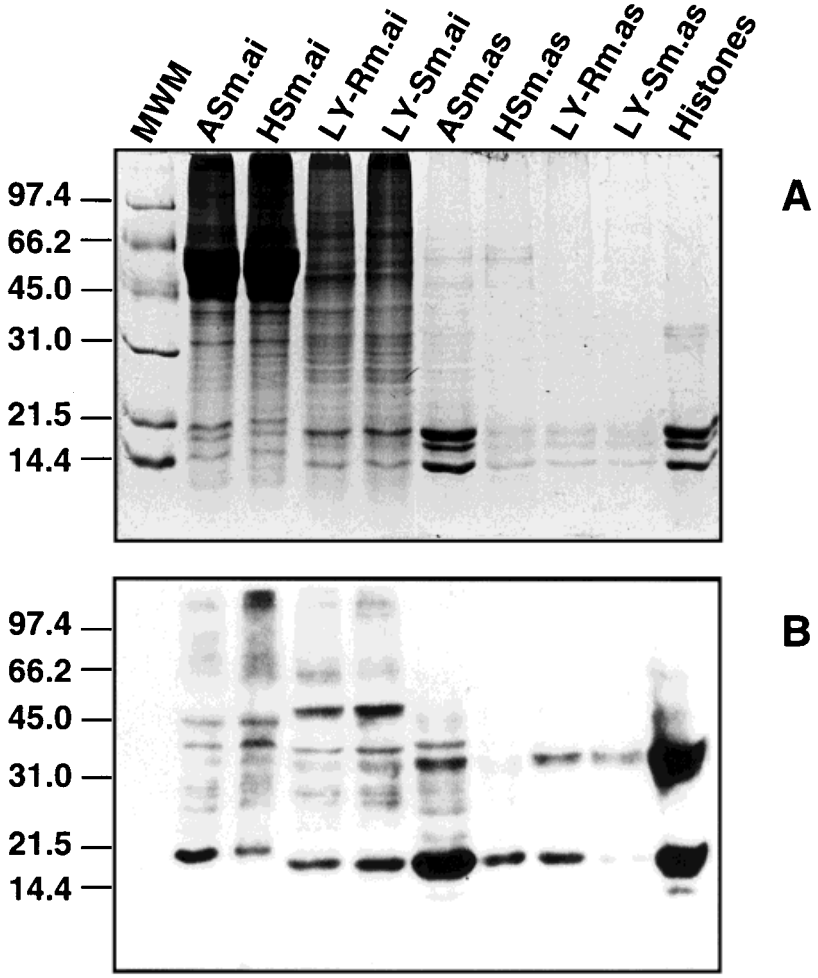

Fig. 3. Poly(ADP-ribose)-binding proteins in acid extracted nuclear matrices. Nuclear matrices (m) were extracted with 0.4N H_2SO_4. Acid insoluble (ai) and acid soluble (as) proteins were loaded on duplicate 10% Proranger gels. After electrophoretic separation, proteins were either stained with Coomassie Blue (**A**) or transferred onto nitrocellulose and subjected to poly(ADP-ribose)-binding assay (**B**). - ASm and HSm: nuclear matrices isolated from HaCaT cells by employing the 0.3 M ammonium sulphate or 2M NaCl (high salt) procedures, respectively. Nuclear matrices from LY-R and LY-S cells were prepared by extracting isolated nuclei with 2M NaCl. Numbers to the left indicate the Mr (KDa) of molecular weight markers (MWM).

Althaus et al., 1998]. Therefore, the stability of poly(ADP-ribose)-nuclear matrix protein complexes was assayed on protein blots by increasing the salt concentration to 1 M NaCl in the washing buffer. Figure 4 shows that under these high stringency conditions, most of the proteins retained their binding to poly(ADP-ribose). Typically, between 20 and 40% of loosely bound radioactivity is removed from polymer-binding proteins by high salt treatment (pending on the type of protein involved). The high stringency treatment also revealed a striking difference in the polymer-binding polypeptide pattern of LY-R and LY-S nuclear matrices: LY-R proteins in the molecular weight range of 45–100 KDa established much stronger interactions with poly(ADP-ribose) than the same components in the LY-S nuclear matrix.

DISCUSSION

A number of studies have identified the nuclear matrix playing an important role in DNA repair [Mullenders et al., 1988; Fernandez and Catapano, 1995; Koehler and Hanawalt, 1996; Bouayadi et al., 1997]. PARP, a DNA nick sensor protein, has been found to be associated with this subnuclear structure [Wesierska-Gadek and Sauermann, 1985; Alvarez-Gonzalez and Ringer, 1988; D'Erme et al., 1990; Golderer et al., 1991; Kaufmann et al., 1991;

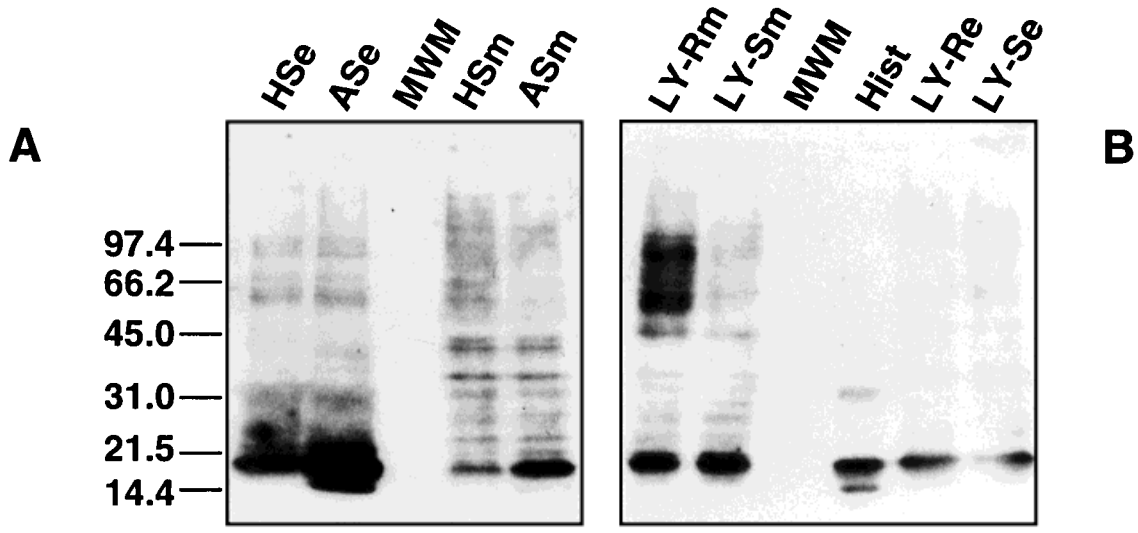

Fig. 4. **A,B:** Salt effect on poly(ADP-ribose)-protein interactions. Following incubation with [^{32}P]-poly(ADP-ribose), the blots shown in Figure 2 were washed with 1M NaCl as described in Materials and Methods. The autoradiograms were exposed three times longer (6 h) than in Figures 2 and 3. The results show a representative experiment out of two matrix preparations.

Quesada et al., 1994]. Recent evidence obtained with transgenic knockout mice shows that the deletion of the PARP gene affects late cellular responses to DNA damage [Mènissier-de Murcia et al., 1997; Wang et al., 1997], suggesting that PARP plays a role downstream of DNA repair events affecting cell survival and cell death pathways. PARP-bound ADP-ribose polymers are likely to constitute the molecular link between the lesion(s) on DNA and pathways downstream of DNA damage processing [Althaus et al., 1998]. Evidence in support of this idea is provided by the *in vitro* observation showing that PARP-bound polymers target three domains of p53 protein, which all contain a 22 aa polymer-binding sequence motif. Polymer-binding to these domains blocks (or reverses) the sequence-specific DNA binding of p53 protein [Malanga et al., 1998].

Our present study shows that nuclear matrix proteins are also potential targets for poly(ADP-ribose)-binding. Such binding occurs both in human and murine cells and presents several levels of specificity: First, only a few out of many proteins in nuclear matrices from mammalian cells are able to bind poly(ADP-ribose). Second, the recovery of poly(ADP-ribose)-binding proteins in nuclear matrix preparations is not dependent on the procedure employed for the isolation of this subnuclear structure. Third, the complexes of nuclear matrix proteins with poly(ADP-ribose) are extremely stable and are not destroyed by high salt treatments.

Of particular interest is the finding that a group of poly(ADP-ribose)-binding matrix proteins in the two closely related mouse lymphoma LY-R and LY-S cells, although exhibiting identical electrophoretic behaviour, strikingly differ in the stability of the complexes they form with ADP-ribose polymers. LY-S cells were derived as an X-ray sensitive, UV-resistant subline from the parental X-ray resistant, UV-sensitive LY-R line [Alexander and Mikulski, 1961]. This spontaneous conversion is accompanied by changes in DNA topology and gene expression. Differential DNA rewinding ability affecting nucleoid sedimentation of irradiated LY-R and LY-S cells has been shown to correlate with radiosensitivity [Kapiszewska et al., 1989; Wlodek and Olive, 1992]. Additionally, differences in the protein composition (Mr range of 28–116 kDa) of nucleoid preparations have been observed. By two-dimensional gel analyses, it has been found that at least nine proteins are missing in nucleoids from LY-S cells [Malyapa et al., 1996]. We also found subtle qualitative and quantitative changes in the polypeptide profiles of the salt-soluble nuclear fractions and nuclear matrices from the two cell sublines. Moreover, differences in the posttranslational modification status could account for differen-

tial polymer-binding to proteins in the Mr range of 45 to 100 kDa (Fig. 4).

Although the individual polymer-binding proteins of the nuclear matrix have not been identified in the present study, it may help further elucidate the complex phenotype of radiosensitive LY-S cells. With the polymer-binding sequence motif at hand, it may become possible to identify a family of nuclear matrix proteins by virtue of their linkage to the PARP-signal pathway. This approach led us in the identification of p53 as a member of the PARP-signal pathway [Malanga et al., 1998]. Another aspect of this speculation is the concept that the radiosensitivity of LY-S cells results primarily from disturbances in DNA damage signaling rather than altered DNA damage processing. The observation that PARP-ko mice are hypersensitive to γ-irradiation while maintaining normal DNA repair proficiency seems compatible with this idea [Ménissier-de Murcia et al., 1997;Wang et al., 1997].

In vitro studies have shown that PARP-bound polymers may recruit signal proteins to DNA strand breakage sites and reprogram some of their domain functions [Althaus et al., 1998]. In fact, an association of PARP with p53, an important DNA damage signal protein, has also been demonstrated in vivo [Wesierska-Gadek et al., 1996]. Deletion of the PARP gene in mammalian cells leads to severe disturbances in the constitutive as well as stress-induced regulation of p53 protein [Agarwal et al., 1997]. The present study reveals another aspect of potentially disturbed signalling, i.e., the alteration of polymer-binding functions in nuclear matrix proteins may affect late responses to DNA damage. An extensive association of endogenous ADP-ribose polymers with nuclear matrix structures has been reported earlier [Cardenas-Corona et al., 1987]. Thus binding of ADP-ribose polymers to selected nuclear matrix proteins may play a physiological role in coordinating DNA strand break signals with pathways downstream of DNA break processing determining the biological expression of DNA damage at the level of cell survival.

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