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ARTICLES

ADP-Ribosylation of Nucleolar Proteins in HeLa Tumor Cells

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Abstract ADP-ribosylation reactions in nucleoli of exponentially growing HeLa cells were studied. Isolated nuclei or nucleoli were labeled with ^{32}P-NAD; then the nucleolar proteins were analyzed by 1-dimensional and 2-dimensional polyacrylamide gel electrophoresis (PAGE) and modified proteins were detected by autoradiography. The labeled nucleolar proteins were also chromatographically fractionated on DEAE-cellulose. Electrophoretic analysis of total nucleolar and chromatographically purified proteins revealed that besides nuclear ADP-ribosyltransferase and histones two characteristic nucleolar phosphoproteins numatrin/B23 and nucleolin/C23 were modified by ADP-ribosylation. © 1993 Wiley-Liss, Inc.

Key words: polyacrylamide gel electrophoresis, ribosomal genes, posttranslational modification

The posttranslational modification of nuclear proteins by attachment of the ADP-ribose has been suggested to play an important role in modulation of chromatin structure, especially in regions of DNA replication, repair, or rearrangement. It is thought to be involved also in other cellular events such as differentiation or transcription in which cleavage and rejoining of DNA strands may occur [for reviews, see Althaus and Richter, 1987; Boulikas, 1991; Gaal and Pearson, 1985; Ueda and Hayaishi, 1985].

The nucleolus is the transcription site of ribosomal genes and here the preribosomal subunits are assembled. Different steps in ribosome biogenesis seem to correspond to distinct nucleolar domains, which can be identified by their morphology. The nucleolus contains a number of proteins, some of them exclusively nucleolar. They seem to play essential roles in transcription, maturation, and packaging of preribosomal particles. One such protein is nucleolin, originally called C23, an abundant protein of 92–100 kD. Numatrin (also called B23 or nucleophosmin) is another major nucleolar protein of about 37 kD [Hernandez-Verdun, 1991; Reimer et al., 1987; Scheer and Bonavente, 1990]. Both proteins are substrates for mitotic cdc2 kinase [Peter et al., 1990] and shuttle between cytoplasm and nucleus [Borer et al., 1989].

Our purpose was to look for nucleolar acceptors of ADP-ribose. This report presents evidence that in dividing HeLa cells two specific nucleolar proteins are modified by ADP-ribosylation.

MATERIALS AND METHODS

Isolation of Nuclei and Nucleoli

HeLa S_3 cells grown at 37°C in suspension culture in Eagle's Minimal Essential Medium supplemented with 10% fetal calf serum were harvested and washed in phosphate-buffered saline (PBS). The cells were swollen and homogenized in 0.25 Nonidet P-40, 0.15% sodium deoxycholate in hypotonic buffer. Crude nuclei were pelleted, resuspended in 0.25 M sucrose, 10 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, and centrifuged through a sucrose cushion. Nucleoli were prepared from isolated nuclei by modification of the methods of Muramatsu et al. [1974] and Rothblum et al. [1977]. The isolated nuclei were suspended in 0.34 M sucrose, 0.5 mM $MgCl_2$, and sonicated until no nuclei remained intact. The sonicate was underlaid with 0.88 M sucrose, 0.5 mM $MgCl_2$, and centrifuged to pellet the purified nucleoli. All isolation steps were performed at 4°C in the presence of protease inhibitors at appropriate concentrations [Zerlauth et al., 1988].

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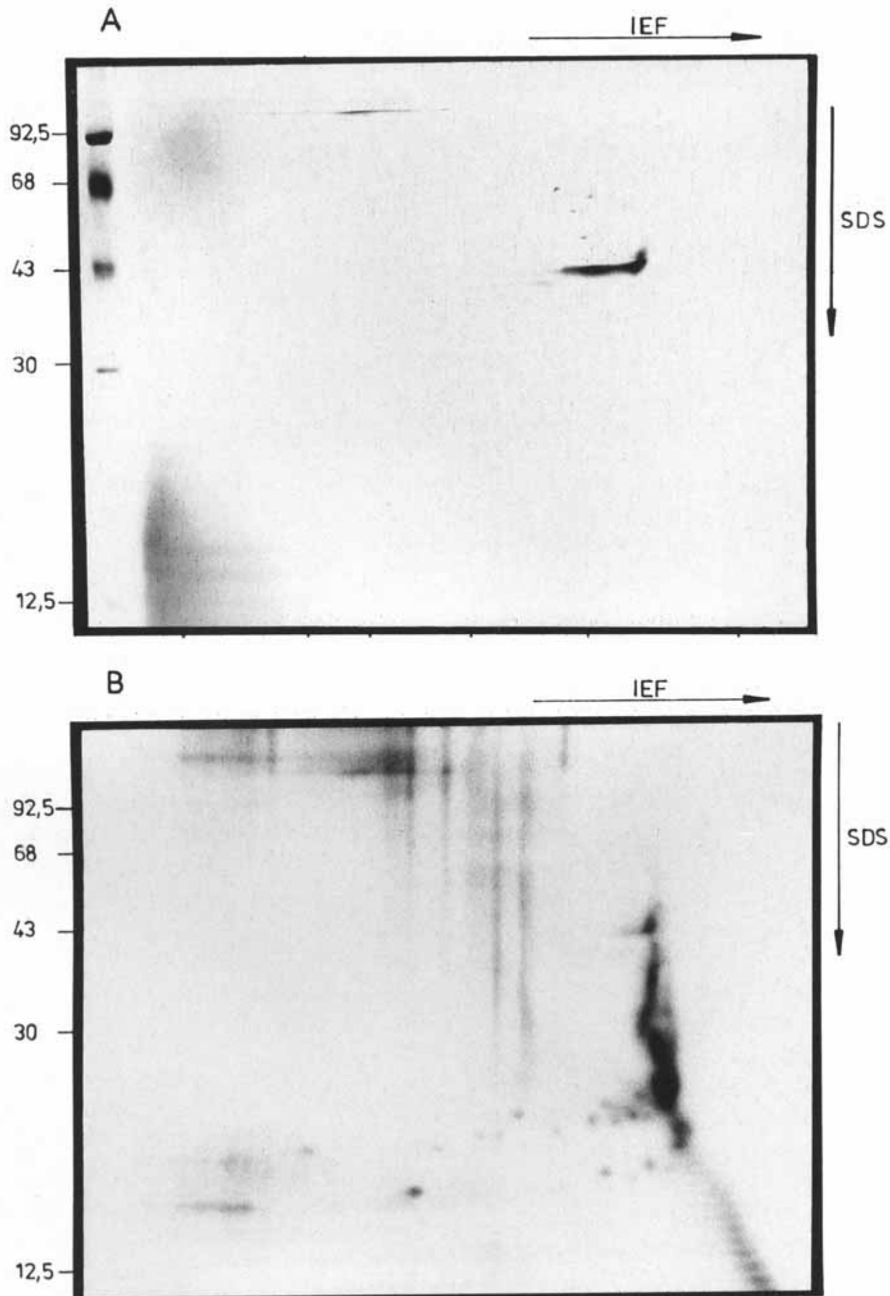

Fig. 1. Analysis of ADP-ribosylated nucleolar proteins by 2D-PAGE Isolated nucleoli were labeled with ^{32}P-NAD and proteins were separated by 2D-PAGE (1st IEF, pH 3 5–10, 2nd 12% SDS-PAGE) 80 μg protein was loaded on the gel **A** Coomassie blue staining **B** Autoradiography

Labeling of ADP-Ribosylated Proteins in Nuclei or Nucleoli

For labeling, the isolated nuclei and nucleoli were incubated with 50 μM or 20 μM (^{32}P)NAD, respectively, in 100 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml DNase I and 10 mM cAMP at 37°C for 30 min. The reaction was terminated by cooling to 0°C.

Quantitative Protein Determination

Protein concentration was determined by the Bio-Rad protein assay using bovine serum albumin as standard.

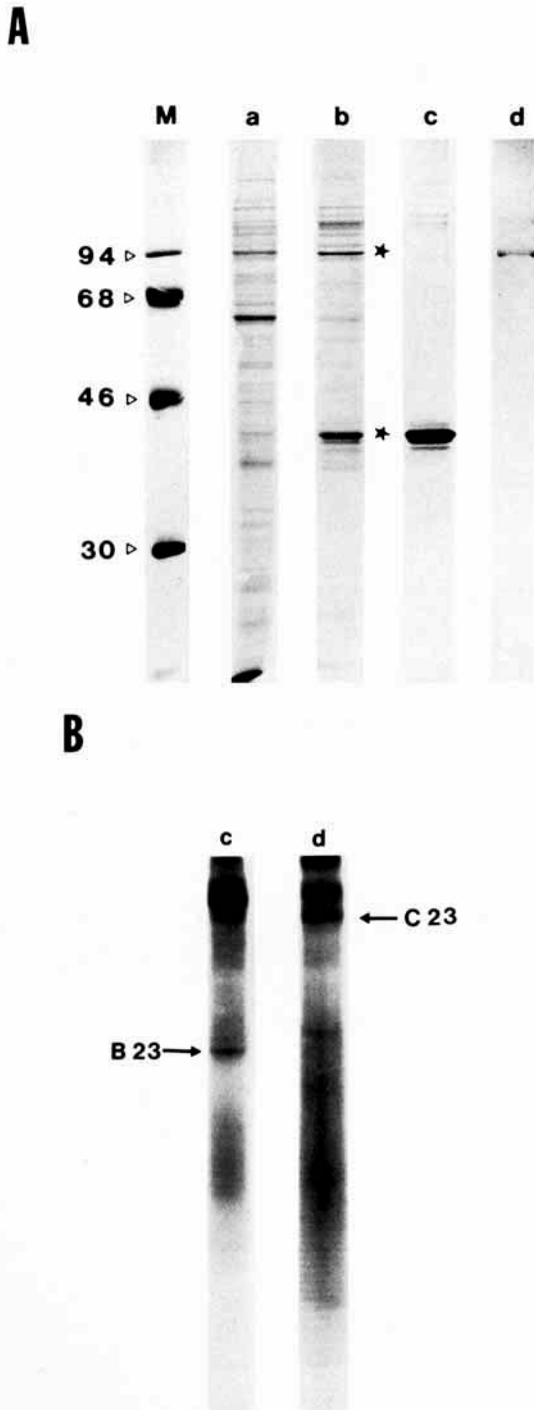

Fig. 2. Chromatographic separation of ADP-ribosylated nucleolar proteins on DEAE-cellulose. A LiCl-urea extract of nucleolar proteins was applied to DEAE cellulose column (DE-52) and fractionated by stepwise elution with increasing NaCl concentration. 10% SDS-PAGE. **A**: Coomassie blue staining. **B**: Autoradiography. **Lane M**: molecular weight marker. **Lane a**: total nucleolar proteins (25 μg). **Lane b**: LiCl-urea extract (20 μg). **Lane c**: 200 mM NaCl eluate from DE-52 (10 μg). **Lane d**: 300 mM NaCl eluate from DE-52 (6 μg).

Purification of Nucleolar Proteins

Isolated nucleoli were treated overnight with 4 M urea, 3 M LiCl. After centrifugation at 27,000*g* for 20 min, the supernatant was dialyzed against 6 M urea, 20 mM Tris-HCl (pH 6.0), 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and again centrifuged. The supernatant containing 2 mg protein was applied to a diethyl aminoethyl (DEAE)-cellulose column (DE-52; Whatman). The proteins were stepwise eluted with increasing NaCl concentrations. The fractions were dialysed, lyophilized, and analysed electrophoretically [Michalik et al., 1981]. Approximately 450 μg protein was not retained on the column and was found in the flow-through fraction. After fractionation on the DEAE-cellulose column, the following amounts of proteins were recovered in each salt fraction: about 340 μg protein in 100 mM NaCl eluate, 290 μg protein in 200 mM NaCl peak, 180 μg in 300 mM NaCl eluate, 210 μg in 400 mM NaCl fraction, and 200 μg in 500 mM NaCl eluate.

Electrophoretic Separation of Proteins

One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10% or 12% slab gels as described by Laemmli [1970]. Additionally, 6 M urea was included in the sample buffer and 4 M urea in the gel solution.

Two-dimensional PAGE was performed according to O'Farell [1975] with modifications as described in detail previously [Wesierska-Gadek et al., 1992]. For calibration IEF protein markers (BDH, Poole, UK), pl range 4.7 to 10.6, were applied. Proteins were detected by Coomassie blue or silver staining. To reduce silver stain background in SDS-PAGE gels, the commonly used cross-linker N,N′-methylene-bis-acrylamide was substituted by piperazine diacrylamide (PDA, Bio-Rad).

Labeled ADP-ribosylated proteins were detected by autoradiography using X-OMAT S film (Kodak, Rochester, NY).

RESULTS

It was our aim to look for ADP-ribose acceptors in the nucleolus. The isolated nuclei were incubated with ^{32}P-NAD and then nucleoli were isolated. Electrophoretic analysis has revealed that some nucleolar proteins were modified (not shown).

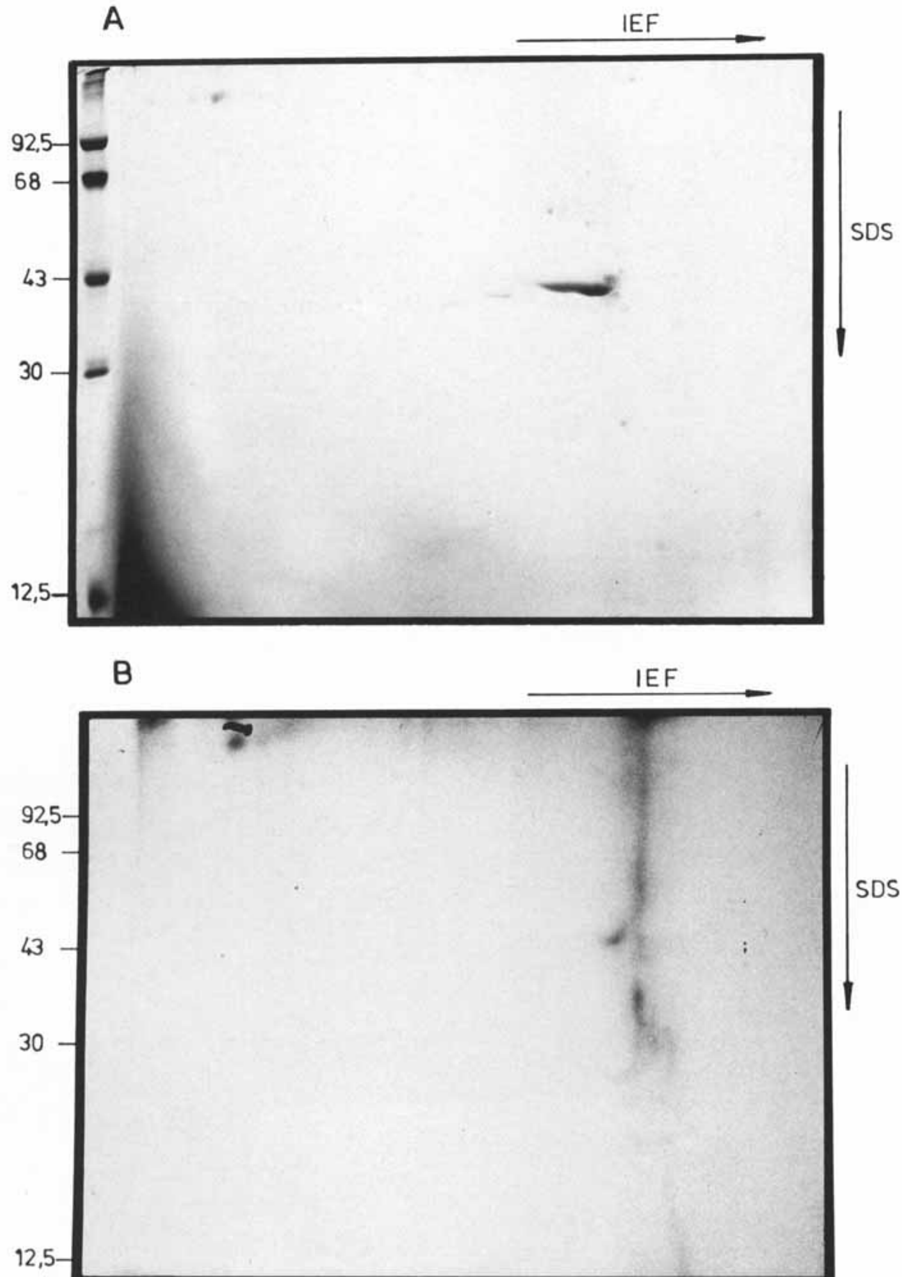

Fig. 3. 2-D PAGE of DEAE-cellulose eluate containing numatrin/B23 50 μg protein was loaded on the gel **A** Coomassie blue staining **B** Autoradiography

To investigate whether endogenous ADP-ribosylating activity is present, isolated nucleoli were directly incubated with radioactive substrate. Indeed, radioactively labeled ADP-ribose was incorporated in nucleolar proteins. Two-dimensional PAGE of this fraction shown in Figure 1 illustrates the pattern of modified proteins. Two intensely Coomassie blue stained proteins were found at about 37 kD, pI 4.8–5.1 and at about 100 kD, pI 5.4–5.6. Corresponding radioactive spots were observed in the autoradiogram. The positions of the stained proteins and their radioactively labeled counterparts coincided with those characteristic for the major nucleolar phosphoproteins numatrin/B23 and nucleolin/C23. Additional radioactive spots were observed in the basic pH range: low molecular weight components presumably representing

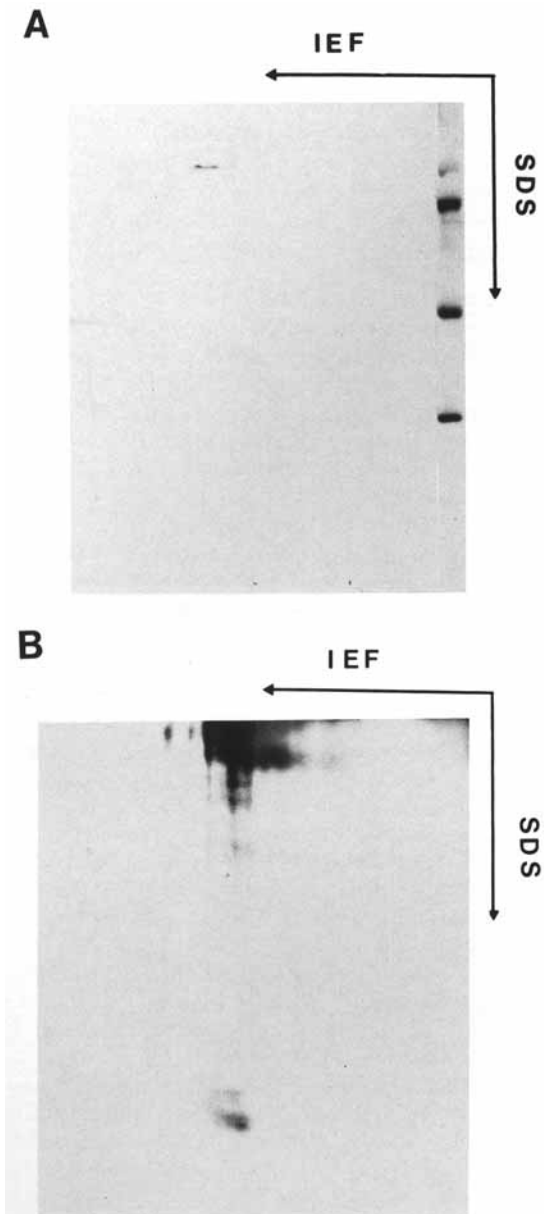

Fig. 4. 2-D PAGE of DEAE-cellulose eluate containing nucleoin/C23. 40 μg protein was loaded on the gel. A: Coomassie blue staining. B: Autoradiography.

modified histones and a labeled protein at about 120 kD corresponding to automodified nuclear ADP-ribosyltransferase.

To further prove the identity of the two labeled proteins found in the acidic pH range, the nucleolar proteins were chromatographically purified on DEAE-cellulose under conditions defined by Michalik et al. [1981]. As shown in Figure 2 numatrin was eluted at 200 mM NaCl and nucleolin at 300 mM NaCl. Electrophoretic analysis of these two fractions revealed the radioactive bands corresponding to numatrin/B23 and nucleolin/C23 (Figs. 2B, 3, 4). To ensure that the ^{32}P was incorporated as ADP-ribose, the labeled proteins were treated with snake venom phosphodiesterase, specific cleaving phosphodiester bonds in ADP-ribose, and with alkaline phosphatase. The labeled residues were sensitive to snake venom phosphodiesterase, but not to alkaline phosphatase, thus confirming that the nucleolar phosphoproteins were modified by ADP-ribosylation.

DISCUSSION

The nuclear ADP-ribosyltransferase, catalyzing the formation of oligo- or polymers of ADP-ribose on a suitable acceptor molecule, has been found almost ubiquitously. The enzyme is mostly localized in chromatin, probably in the internucleosomal space [Mullins et al., 1977]. Nuclear ADP-ribosyltransferase has also been found to be associated with the nuclear matrix [Wesierska-Gadek and Sauermann, 1985]. Contradictory findings have been reported on the occurrence of the enzyme in nucleoli [Hilz and Kittler, 1968, versus Kawashima and Izawa, 1981].

Concerning the localization of the enzyme we have found in this study that the ADP-ribosyltransferase activity is present in isolated nucleoli of HeLa cells. The specific activity of the nucleolar enzyme was comparable to that in nuclei, indicating that the nucleolar enzymatic activity was not due to chromatin contamination. The nucleolar ADP-ribosyltransferase also modified specific nucleolar proteins.

Until now a number of nuclear proteins have been identified as substrates for ADP-ribosylation. In particular histones have been well characterized as acceptors. The main nonhistone proteins known to be modified are nuclear ADP-ribosyltransferase itself and high mobility group (HMG) proteins. It is noteworthy that basic proteins are especially prone to be ADP-ribosylated. In this context it is interesting that two negatively charged nucleolar phosphoproteins, numatrin and nucleolin, are good acceptors for ADP-ribose.

Both nucleolar phosphoproteins are present in large amounts in nucleoli with active ribosomal biogenesis and are widely distributed in higher eukaryotes. Nucleolin, which binds to nascent rRNA, is thought to participate in the early steps of ribosome biogenesis, such as regu-

lation of the transcription by RNA polymerase I and modulation of the chromatin structure in the nucleolus [Hernandez-Verdun, 1991]. Numatrin binds with high affinity to single-stranded nucleic acids, and exhibits RNA-helix destabilizing activity. This protein, associated with the most mature nucleolar preribosomal RNP, appears to be involved in the later stages of ribosome assembly [Hernandez-Verdun, 1991]. Both proteins were found to shuttle between nucleolus and cytoplasm [Borer et al., 1989].

The present study demonstrates the novel modification of numatrin and nucleolin. Our observations point to a regulatory function of ADP-ribosylation in nucleolar processes. The detailed role of such protein modification remains, however, to be elucidated.

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