DEMONSTRATION OF IN VITRO COVALENT MODIFICATION OF CHROMOSOMAL PROTEINS BY POLY(ADP) RIBOSYLATION IN PLANT NUCLEI

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1. Introduction

Chromosomal proteins are known to undergo a series of different post-translational modifications, such as methylation, phosphorylation, acetylation and poly(ADP) ribosylation. Some of these events have been correlated to gene activation (histone acetylation) [1] or to changes in the chromatin condensation during the life cycle of a cell (histone phosphorylation) [2]. Since the first report [3] on the formation of poly(ADP-ribose) in isolated nuclei, numerous reports on this covalent modification of histones and nonhistone chromosomal proteins have appeared and a correlation with DNA synthesis and cell differentiation was suggested [4].

Although there are two preliminary reports of the occurrence of poly(ADP-ribose) in plants [5,6], the covalent incorporation of ADP-ribose into chromosomal proteins was only observed in nuclei or chromatin from animal tissue; there is no evidence whether this modification can also occur in nuclei of the plant kingdom [7]. This may be a corollary of the greater difficulties in the isolation of highly purified and physiologically active nuclei from plant tissue. Based on a new method developed for the isolation of nuclei from plant tissue culture cells (L.W., in preparation), this report describes for the first time the covalent modification of chromosomal proteins by poly(ADP-ribose) in plant nuclei obtained from transformed tissue cultures of *Nicotiana tabacum*. The results show a strong similiarity of this nuclear protein modifying activity to those described in animal systems.

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2. Materials and methods

[*Carbonyl*-^{14}C]NAD^+ was purchased from Amersham Buchler, [*adenylate*-^{32}P]NAD^+ was obtained from New England Nuclear; snake venom phosphodiesterase, deoxythymidine and nicotinamide are products from Boehringer, Mannheim, RNase A and DNase I were purchased from Sigma-Chemie, München.

Cellulase and pectinase from *Aspergillus niger* (Serva Feinbiochemica) were purified by dissolving in 0.5 M acetic acid, centrifugation and gel filtration of the supernatant upon Bio-Gel P6 and subsequent lyophilisation.

2.1. *Cell cultures*

Two different cultures from *Nicotiana tabacum* var. White Burley were used which were transformed by the *Agrobacterium tumefaciens* strains A6 and B6S3, respectively. These cultures were originally obtained from Dr R. Schilperoort, Leiden. Cultures were grown in the media of [8] without phytohormones at 25°C and subcultured every week. Nuclei were prepared from cells 3–4 days after subculturing in mid-log phase.

2.2. *Isolation of nuclei*

The detailed procedure will be described elsewhere. In brief, nuclei were isolated from ~0.5–1 kg plant cells as follows: After a short plasmolysis in 0.7 M mannitol, 10 mM MES (pH 5.8), 0.1% bovine serum

albumin and subsequent incubation for 30 min at 25°C with 0.1% (w/w) of the purified cellulase–pectinase preparation, cells were washed with isolation buffer (10 mM NaCl, 10 mM MES (pH 6.0), 3 mM $MgCl_2$, 0.1 mM EDTA, 20 mM mercaptoethanol, 0.25 M sucrose, 0.2 mM PMSF, 1 μM Pepstatin, 0.6% Nonidet P 40) and homogenized with a motor-driven mortar and pestle in the same medium. After filtration nuclei were purified from cell wall residues and cytoplasmic contamination by differential centrifugation and subsequent isopyknic centrifugation using Perkoll (Pharmacia Fine Chemicals) as density gradient medium, in the presence of PMSF, pepstatin, and EDTA to inhibit proteases.

2.3. *Preparation of histones*

For the preparation of histones, nuclei were washed after incubation (cf. fig.1) with a buffer consisting of 5% glycerol, 0.1 mM EDTA, 10 mM $MgCl_2$, 10 mM MES (pH 6.0) and 5 mM DTT until the radioactivity in the supernatant was negligible. Histones were then extracted with 0.4 N H_2SO_4, precipitated by the addition of 5 vol. acetone and lyophilized.

3. Results and discussion

Figure 1 shows the incorporation of [^{14}C]ADP-ribose into the acid-precipitable fraction of tobacco nuclei at 25°C. The reaction reaches a plateau after ~20 min and is strongly inhibited by low concentrations of either deoxythymidine (3 mM) or nicotinamide (3 mM). The inhibitory action of both substances exhibits a strong resemblance to animal systems where thymidine derivatives as well as nicotinamide were shown to inhibit the poly(ADP) ribosylating activity [9]. Furthermore this inhibition demonstrates that the formation of trichloroacetic acid-precipitable activity is an enzymatic reaction and not a chemical reaction which has been discussed as a possible pitfall in animal systems [10].

As shown in table 1 radioactivity is neither solubilized by DNase nor RNase treatment, thus providing evidence that the acid-insoluble radioactivity is not incorporated into nucleic acids. Upon treatment with snake venom phosphodiesterase and subsequent chromatography on PEI-cellulose plates two radioactive spots could be distinguished corresponding

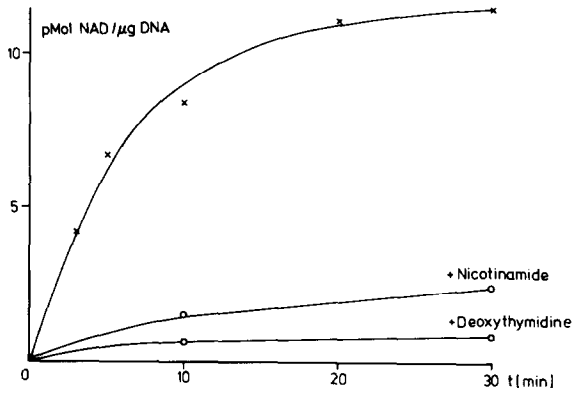

Fig.1. Kinetics of the incorporation of [^{14}C]NAD into poly-(ADP-ribose) by isolated nuclei. Inhibitory action of 3 mM deoxythymidine and 3 mM nicotinamide. Aliquots (0.4 ml) of the purified nuclei suspension (containing ~100 μg DNA) were incubated at 25°C for different times in 60 mM Tris–HCl (pH 7.9), 0.1 mM EDTA, 5 mM dithiothreitol, 10 mM $MgCl_2$, 5% glycerol and 0.2 mM [^{14}C]NAD (2 μCi). The reaction was terminated by adding 20% trichloroacetic acid (TCA). Acid-soluble material was filtered on glassfibre filter (GF/C, Whatman), washed twice with 20% TCA and once with ethanol.

to AMP and phosphoribosyl-AMP. Figure 2 shows the radiochromatogram of a typical separation. Hence the incorporation of NAD^+ into acid-precipitable material is due to the formation of poly(ADP-ribose). The chainlength was estimated according to [11] by integrating the peaks of the radiochromatogram corresponding to AMP and phosphoribosyl-AMP. In several independent experiments chainlengths between

Table 1
Acid-insoluble radioactivity is resistant to DNase and RNase treatment

Incubation with	Trichloroacetic acid-precipitable		Trichloroacetic acid-soluble	
	cpm	%	cpm	%
DNase I	26020	93	1902	7
RNase A	23457	94	1450	6
Water	23469	93	1683	7

^{14}C-Labeled nuclei were incubated for 90 min at 37°C with either DNase I (0.4 mg/ml) in 0.2 M sodium acetate/20 mM $MgCl_2$ (pH 5.0) or RNase A (0.4 mg/ml) in 0.2 M sodium acetate (pH 5.0). Acid-precipitable material was dissolved in Soluene 350 (Packard) before counting

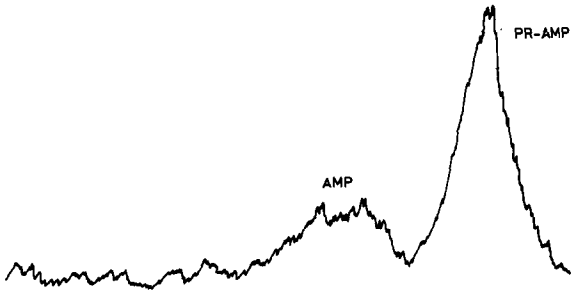

Fig.2. Detection of AMP and phosphoribosyl-AMP (PR-AMP) after digestion of acid-insoluble ^{14}C-labeled material with snake venom phosphodiesterase according to [19]. Products obtained were separated on polyethyleneimine cellulose thin-layer sheets which were developed first in methanol, then in 1.0 M acetic acid and finally in 0.9 M acetic acid/0.3 M LiCl. After drying, radioactive spots were detected with a radiochromatogram scanner (Hewlett-Packard).

3.4 and 8.6 were obtained, i.e., the same range as found in animal systems [4] (cf. table 2). After incubation of the labeled products with 0.1 N NaOH or neutral hydroylamine at 37°C for 30 min (table 3) >80% of the radioactivity became acid-soluble. This suggests the existence of a covalent linkage between the poly(ADP-ribose) and its acceptors, i.e., proteins (cf. fig.3). The lability towards neutral hydroxylamine supports the assumption that the poly(ADP-ribose) moiety is bound to a carboxyl group of the acceptor protein [12].

The covalent linkage of the poly(ADP-ribose) with chromosomal proteins could be confirmed by the following experiment: one aliquot of histones, extracted as in section 2, was treated with 1 M NH_4OH at 37°C for 30 min and subsequently lyophilized, whereas a

Table 2
Average chain length of poly(ADP-ribose)

Expt.	Relative amount		Chain-length
	AMP	PR-AMP	
1	14	108	8.2
2	17	90	6.3
3	36	90	3.5

Chainlength was determined by integrating the areas of the radiochromatogram corresponding to AMP and PR-AMP:

Chainlength = (AMP + PR-AMP)/AMP

Table 3
Sensitivity of poly(ADP-ribose) linkage to NaOH and neutral NH_2OH

Incubation with	Trichloroacetic acid-precipitable		Trichloroacetic acid-soluble	
	cpm	%	cpm	%
0.1 N NaOH	4309	17	19 609	83
0.7 M NH_2OH	4504	17	20 605	83
Water	17 205	97.9	362	2.1

^{14}C-Labeled material was incubated with either 0.1 N NaOH or 0.7 M NH_2OH (pH 7.0) for 30 min at 37°C

second aliquot was treated with distilled water. Both samples were subsequently subjected to SDS slab gel electrophoresis. Figure 3 shows a direct comparison of the stained gels and the corresponding autoradio-

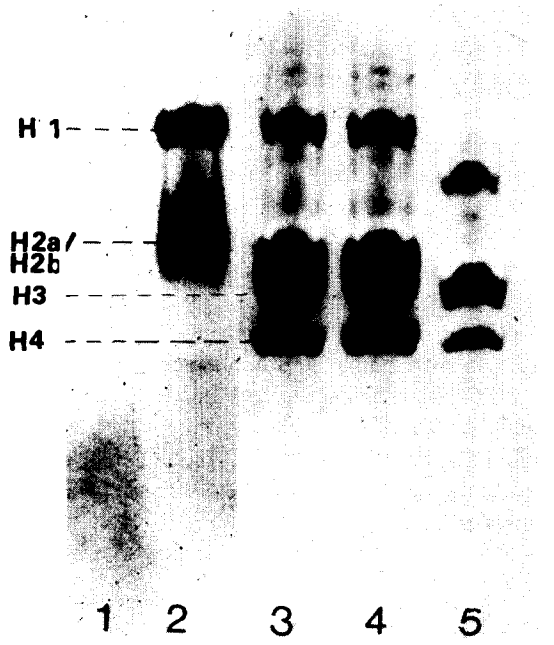

Fig.3. Demonstration of the covalent modification of histones H1 and H2a/H2b by poly(ADP-ribose). Histones extracted as in section 2 were subjected to SDS-gel electrophoresis at pH 6.8. The rather low pH was necessary in order to prevent splitting of the poly(ADP-ribose) from the proteins which occurs during alkaline pH. Lane 1 (autoradiogram) and lane 3 (stain) show the histones, which have been treated with 1 M NH_4OH for 30 min at 37°C; lane 2 (autoradiogram) and lane 4 (stain) show the nontreated histones. Lane 5 shows the stain of calf thymus histones for comparison.

grams. No radioactivity can be detected in the radioautogram of the NH_4OH-treated histones (lane 1) compared to the radioautogram of the nontreated sample (lane 2). That this is not due to breakage of proteins during NH_4OH treatment shows a comparison of lane 3 (stain of NH_4OH treated histones) with lane 4 (stain of untreated histones). Thus poly(ADP-ribose) is covalently attached to proteins and not bound by any unspecific adsorption. Furthermore a comparison of lane 2 and lane 4 reveals that the main acceptors for poly(ADP-ribose) among the histones are the lysine-rich histone H1 and the moderately lysine-rich histones H2a/H2b (also called plant histones PH1 and PH2). No modification of the arginine-rich histones H3 and H4 can be detected. Whereas the preferential modification of histone H1 has been found in most animal systems described, the modification of the H2a/H2b fraction is more controversial [13–18]. It is of special interest, that a modification of H2a/H2b is found in these plant nuclei; they represent the least conserved species of the core histones. There is no modification in the highly conserved histone pair H3/H4.

In conclusion this report describes for the first time the existence of a poly(ADP) ribosylating activity in the chromosomal proteins of plants. As far as investigated this system shows a striking similarity to the animal systems previously described concerning the action of several inhibitors, the average chain-length, the fact that >80% of the poly(ADP-ribose) are covalently bound to protein acceptors, the nature of this covalent linkage as based on its instability in neutral hydroxylamine and even the main acceptors among the histone species, i.e., H1 and H2a/H2b. It remains to be investigated whether the activity of the poly(ADP) ribosylating system is correlated in the same way with the cell cycle as found for animal systems.

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