ADP-ribosylation of nuclear proteins in mouse testis

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The nuclear acceptor proteins for poly(ADP-ribose) were investigated in mouse liver and testis. In liver, histones are ribosylated preferentially, whereas in testis the major acceptors are non-histone proteins. An analysis of the purified testicular acceptor proteins suggests that they are high- and low-mobility-group-like proteins.

In addition to phosphorylation and methylation, ADP-ribosylation is an important mechanism for modification of cellular proteins (reviewed by Hayaishi & Ueda, 1977). The transfer of ADP-ribose from NAD to protein and the formation of a homopolymer of ADP-ribose units are catalysed by poly(ADP-ribose) synthetase, an enzyme found predominantly in the nucleus of eukaryotic cells. ADP-ribosylation of nuclear proteins has been correlated with a variety of regulatory functions, such as DNA synthesis (Burzio & Koide, 1973), DNA repair (Durkacz *et al.*, 1980), chromatin structure (Lorimer *et al.*, 1976), gene expression (Müller & Zahn, 1976) and cell differentiation (Hilz & Stone, 1976). However, a fundamental problem still unresolved concerns the identity of the nuclear acceptor proteins. Histones (Ueda *et al.*, 1975; Ord & Stocken, 1977; Wong *et al.*, 1977) and non-histone proteins (Giri *et al.*, 1978; Mullins *et al.*, 1977; Minaga *et al.*, 1979) have all been reported to contain ADP-ribose, either as a monomer or as a single or branched chain. These discrepancies may be explained in part by variations in the methodology used to label and extract protein–(ADP-ribose) (discussed by Minaga *et al.*, 1979), and in part by the fact that there may be genuine differences between tissues. In this investigation we have compared, using the same extraction and chromatographic procedures, the type of nuclear proteins ADP-ribosylated *in vivo* in mouse liver and testis.

Abbreviations used: SDS, sodium dodecyl sulphate; LMG proteins, low-mobility-group proteins; HMG proteins, high-mobility-group proteins.

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Materials and methods

Materials

All chemicals and enzymes were of the highest purity available commercially and were obtained from Sigma (London) or BDH. The Radiochemical Centre, Amersham, Bucks., U.K., supplied D-[1-¹⁴C]ribose (60 Ci/mol), [8-³H]adenine (15–25 Ci/mol) and nicotinamide-[U-¹⁴C]adenine dinucleotide (280–300 Ci/mol). Molecular-weight marker proteins were obtained from Pharmacia (G.B.) Ltd., Hounslow, Middx., U.K. CM-cellulose 32 was purchased from Whatman Biochemicals, Maidstone, Kent, U.K., and AG1-X2 resin (200–400 mesh, 2% cross-linkage) from Bio-Rad Laboratories, 20090 Segrate, Milan, Italy.

Labelling, extraction and analysis of nuclear proteins

Adult male mice (CFLP strain) weighing 30–40 g were anaesthetized with diethyl ether and injected either intraperitoneally with a mixture of 25 μ Ci of [¹⁴C]ribose and 50 μ Ci of [³H]adenine (for labelling liver), or intratesticularly with 5 μ Ci of [¹⁴C]ribose and 5 μ Ci of [³H]adenine. Preliminary experiments showed that maximum incorporation of labelled precursors into acid-insoluble protein was reached after 1 h in the liver and after 2 h in the testis. At the appropriate time interval, liver and testes were removed, homogenized immediately in 3 vol. of ice-cold 5% (v/v) HClO₄ and centrifuged at 10 000 g for 20 min. Precipitated proteins were washed three times in 5% HClO₄ at 4°C and then extracted with 0.25 M-HCl as described by Ueda *et al.* (1975). The HCl-extracted proteins were chromatographed on CM-cellulose 32 (1.5 cm $\times$ 17.0 cm; Ueda *et al.*,

1975) and AG1-X2 (formate form; 0.9 cm × 10.0 cm) columns, with a linear gradient from 0.005 to 0.01 M-formic acid.

Proteins were analysed either on 15% (w/v) polyacrylamide slab gels at pH 2.9 as described by Goodwin *et al.* (1973), or on 15% polyacrylamide slab gels containing 1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol (Jones *et al.*, 1980).

Purified proteins were hydrolysed in 6 M-HCl at 110°C for 20 h, and released amino acids were analysed on an LKB (model 4400) amino acid analyser.

Digestion with snake venom phosphodiesterase and analysis of products

A portion of the HCl-extract was neutralized with KOH and incubated for 48 h at 25°C with snake venom phosphodiesterase (100 µg/ml) in 0.25 M-Tris/acetate buffer, pH 8.8, containing 0.025 M-MgCl₂. The digestion was terminated by the addition of HClO₄ (final concn. 5%), the mixture was centrifuged (5000 g for 30 min), and the supernatant fraction chromatographed on a AG1-X2 (formate form) column as described by Ueda *et al.* (1975).

Other procedures

The activity of poly(ADP-ribose) synthetase in whole homogenates of mouse testis and liver was measured as described previously (Farina *et al.*, 1979). Total protein was measured by a modified Lowry procedure (Bensadoun & Weinstein, 1976), with bovine serum albumin as standard.

Results

The activity of poly(ADP-ribose) synthetase in mouse testis was found to be 0.30 munit/mg of protein, as opposed to 0.18 munit/mg of protein in liver. From 1 g of labelled testis tissue, 15.0 mg of protein was recovered in the HClO₄-insoluble HCl-extractable fraction (a mixture of histones and non-histone proteins), with a specific radioactivity of 7840 c.p.m./mg. On the other hand, 1 g of liver yielded 18.7 mg of protein in the HCl extract, with a specific radioactivity of 4010 c.p.m./mg. When the HCl-extractable proteins from testis were separated by chromatography on CM-cellulose 32, 90% of the total radioactivity recovered was present in the first (non-histone) protein peak (Fig. 1*a*). The remaining radioactivity was eluted mostly in a position corresponding to histone H1, a small amount being also present in peaks corresponding to histones H2 and H3. By contrast, in liver only 13% of the total radioactivity recovered was present in the non-histone peak, but 57% was associated with the histone-H1 peak, 24% with histones H2 and 6% with the peak corresponding to histone H3 (Fig. 1*b*).

To demonstrate that the radioactivity present in

Fig. 1. Chromatography on CM-cellulose 32 of HClO₄-insoluble HCl-extractable proteins labelled *in vivo* with [¹⁴C]ribose and [³H]adenine: (a) testis; (b) liver. Proteins were loaded on the column in 0.1 M-sodium acetate buffer, pH 4.2, followed by stepwise elution with 0.17 M-acetate buffer, pH 4.2, containing (A) 0.42 M-NaCl, (B) 0.01 M-HCl and (C) 0.02 M-HCl respectively as indicated by the arrows. The column was calibrated with known histone standards (H1 etc.) Protein was measured by A₂₃₀ (—) and total radioactivity (---) by counting portions of each fraction in 5 ml of Lumagel scintillation fluid in a Beckman scintillation counter (model LS8100).

the HCl extract from the testis was in the form of ADP-ribose covalently bound to protein, the HCl extract was digested with snake venom phosphodiesterase and the acid-soluble products were analysed on a AG1-X2 (formate form) column (Ueda *et al.*, 1975). The enzyme treatment solubilized approx. 55% of the bound radiolabel, and the major peaks eluted from the AG1-X2 formate column co-chromatographed with authentic 5'-AMP and ADP-ribose standards (results not shown).

An analysis of the total HCl extract from testis on 15% polyacrylamide gels at pH 2.9 revealed a complex mixture of proteins consisting mainly of histones and LMG proteins (Fig. 2*a*, track ii). The first peak from the CM-cellulose column (containing most of the bound radioactivity) comprised two major basic proteins (Fig. 2*a*, track iii). When a parallel track containing these proteins was excised from the gel containing SDS, it was found that the most cationic protein had a mol.wt. of approx.

Fig. 2. Electrophoresis of HCl extract and purified acceptor proteins from testis: (a) non-denaturing polyacrylamide gel at pH 2.9; (b) denaturing polyacrylamide gel containing SDS

Track (i), a mixture of calf thymus histone standards; track (ii), total HCl extract; track (iii), non-histone proteins from CM-cellulose; track (iv), first radioactive peak from AG1-X2 (Fig. 3); track (v), third radioactive peak from AG1-X2 (Fig. 3).

Table 1. Amino acid composition (mol %) of purified ADP-ribose acceptor proteins from mouse testis

Non-histone acceptor proteins for ADP-ribose were purified by chromatography on CM-cellulose and AG1-X2 (formate form) columns, and amino acids analysed as described in the Materials and methods section. Values for aspartic acid and glutamic acid include asparagine and glutamine respectively. Abbreviation: ND, not-detectable.

Amino acid	20000- mol.wt. protein	70000- mol.wt. protein
Lysine	11.6	6.8
Histidine	3.3	1.5
Arginine	4.4	6.1
Aspartic acid	11.1	12.6
Threonine	4.6	5.5
Serine	7.8	11.3
Glutamic acid	13.8	10.8
Proline	ND	ND
Glycine	10.4	14.4
Alanine	8.2	13.2
Valine	6.9	5.2
Methionine	Trace	Trace
Isoleucine	2.5	2.7
Leucine	7.1	6.0
Tyrosine	3.9	6.0
Phenylalanine	3.9	2.3
Lysine : arginine	2.6	1.1
Basic amino acids (%)	20.0	14.7
Acidic amino acids (%)	24.9	23.4

Fig. 3. Chromatography on a AG1-X2 (formate form) column of the non-histone protein fraction from testis

Fractions 1–50 from Fig. 1(a) were pooled, dialysed against 5 mM-formic acid and applied to a AG1-X2 (formate form) column. Proteins were eluted with a linear gradient of 5–10 mM-formic acid (●). Protein (A_{230m} —) and radioactivity (----) were monitored in fractions as described in Fig. 1.

detected on polyacrylamide gels with Coomassie Blue, but the first and third peaks consisted primarily of the 20000- and 70000-mol.wt. proteins respectively (Fig. 2b). Both proteins were over 90% homogeneous, as determined from densitometric scans of the gels. An amino acid analysis of these proteins (Table 1) revealed that the 20000-mol.wt. protein had a lysine:arginine ratio of 2.6:1 and basic amino acids comprised 20% of the total, whereas the analogous values for the 70000-mol.wt. protein were 11:1 and 14.7% respectively. Together with their respective mobilities on polyacrylamide gels at pH 2.9, these results support identification of the 20000- and 70000-mol. wt. proteins as HMG- and LMG-like respectively.

Discussion

Results on the nature of nuclear acceptor proteins for poly(ADP-ribose) should be treated with some caution, since allowances must always be made for inadequate extraction of other chromatin-associated proteins and/or their degradation during analysis. It is known that the protein-(ADP-ribose) bond is very labile at neutral or alkaline pH (Minaga *et al.*, 1979), and that *in vivo* the polymer undergoes fairly rapid metabolism (Kun *et al.*, 1975).

20000 and the least cationic a mol.wt. of approx. 70000.

To purify the above proteins, the non-histone fraction collected after chromatography of the HCl extract from testis on CM-cellulose was fractionated further on a AG1-X2 (formate form) column (Fig. 3). Approx. 55% of the protein and 66% of the total radioactivity were eluted immediately after the void volume. Two other radioactive peaks, one eluted before and one after the application of the gradient, were also detected. The second peak of radioactivity was associated with too little protein for it to be

However, Minaga *et al.* (1979) have shown that rapid extraction with HClO_4 and HCl at $0-5^\circ\text{C}$ minimizes decomposition of the poly(ADP-ribose) chain. The present results on mouse liver, by using dilute mineral acids to extract nuclear proteins, are in good agreement with those reported previously by Ueda *et al.* (1975) on rat liver; in both cases histone H1 is the major nuclear acceptor protein. Using the same extraction protocol employed for liver, we have found that in testis non-histone proteins are ribosylated preferentially to such an extent that less than 10% of the incorporated $[^{14}\text{C}]\text{ribose} + [^3\text{H}]\text{adenine}$ is associated with histones. Similar results have been obtained when purified nuclei from mouse testis are incubated with labelled precursors *in vitro*, confirming that the ADP-ribosylated proteins are of nuclear origin. Therefore there are real differences between tissues in the type of nuclear acceptor proteins for ADP-ribose.

An analysis of the non-histone fraction from testis revealed two proteins which, from their electrophoretic mobility, their content of basic and acidic amino acids, and their ratio of lysine:arginine residues, resemble HMG and LMG proteins. The HMG-like protein, mol.wt. 20000, has properties in common with HMG proteins 1, 2, 14 and 20 from calf thymus (Goodwin *et al.*, 1973; Walker *et al.*, 1978) and also with HMG-T, a nuclear protein specific to trout testis (Watson *et al.*, 1977). The amino acid composition of the 70000-mol.wt. protein is also similar to the only previously analysed LMG protein from calf thymus (Goodwin *et al.*, 1973).

It has been estimated that HMG proteins comprise approx. 10^6 molecules per nucleus (about 10% of the amount of histones), but it is not known if they can modulate gene activity. Wong *et al.* (1977) and Levy-Wilson (1981) have shown in trout testis nuclei that proteins HMG-T and H6 are ADP-ribosylated, and that these HMG proteins are localized preferentially in the transcriptionally competent regions of chromatin. On the basis of this, Levy-Wilson (1981) has speculated that because testis tissue has a high rate of cell division, ADP-ribosylation of HMG proteins may be involved in DNA replication and repair. Another possible role would be in condensation of chromatin into the spermatozoa nucleus. However, we do not know whether the poly(ADP-ribose) synthetase is located primarily in Leydig cells or in the spermatogenic epithelium, or if it is associated with a specific developmental stage of spermatozoa. Since the testis is a target organ for

pituitary gonadotropins, it is also possible that ADP-ribosylation of HMG proteins is involved in regulating gene activity. Because of the high specificity with which the HMG and LMG proteins are ADP-ribosylated, the testis should serve as a good tissue for investigating the effects of this process on these proteins.

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