Nucleolar Specific Acidic Phosphoprotein C23 Is Highly Methylated*

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Protein C23 (Mr = 110,000; pl, 5.5) is the major phosphoprotein in the nucleolus of Novikoff hepatoma cells and comprises 9.5% of the total nucleolar protein. In addition to being highly phosphorylated (1.2 mol % of phosphoserine), it is also highly methylated. Protein C23 contains 1.3 mol % of \( \text{N}^\text{V}, \text{N}^\text{VI} \)-dimethylarginine and a trace of \( \text{N}^\text{VI} \)-monomethylarginine.

The nucleolus of Novikoff hepatoma cells contains two major acidic phosphoproteins, protein C23 (Mr = 110,000; pl, 5.5) and protein B23 (Mr = 37,000 pl 5.5) (1–5). These proteins have been immunohistochemically localized specifically to the nucleolus (1, 4). Protein C23 was also found to be concentrated in the fibrillar centers of nucleoli and present on the nucleolus organizer regions of metaphase chromosomes (1). It has also been proposed that protein C23 is, in part, responsible for silver uptake at the nucleolus organizer regions (3). The presence of protein C23 on the nucleolus organizer regions illustrates its association with ribosomal gene chromatin. This spatial relationship suggests that it is an essential component of the pre-rRNA synthesizing machinery.

Protein C23 has been isolated by preparative polyacrylamide gel electrophoresis (2) and by ion exchange column chromatography (1). The purified protein was found to be highly phosphorylated, it contains 1.2 mol % of phosphoserine (5). The sequence of one acidic tryptic phosphopeptide has been reported (2). A similar protein isolated from mouse ascites sarcoma cell nuclei has been partially characterized (6, 7).

In this report, data is presented which shows that protein C23 is subjected to the post-translational modification of methylation in addition to phosphorylation. Protein C23 contains 1.3 mol % of \( \text{N}^\text{V}, \text{N}^\text{VI} \)-dimethylarginine and a trace of \( \text{N}^\text{VI} \)-monomethylarginine.

MATERIALS AND METHODS

\( \text{N}^\text{V}, \text{N}^\text{VI} \)-Dimethylarginine, \( \text{N}^\text{V}, \text{N}^\text{VI} \)-dimethylarginine, and \( \text{N}^\text{VI} \)-monomethylarginine were purchased from Calbiochem. L-[methyl-\( \text{H} \)] Methionine (54 mCi/mmol) was obtained from New England Nuclear. Novikoff hepatoma tissue culture cells (N.S.7-3) were grown in Dulbecco’s minimum essential medium with 5% fetal calf serum and antibiotics. To label the methylated arginine residues, the cells were incubated for 1 h in methionine-free medium and then for 4 h in medium containing 1-[methyl-\( \text{H} \)]methionine. The cells were harvested and combined with Novikoff hepatoma ascites cells. Nucleoli were isolated as previously described (8). Protein C23 was isolated by DEAE-cellulose and Bio-Rad AG5-X4A column chromatography as reported (1).

Polyacrylamide Gel Electrophoresis—The one-dimensional sodium dodecyl sulfate-polyacrylamide gel system used was that of Laemmli (9). The nucleoli were suspended in sodium dodecyl sulfate sample buffer, heated for 3 min at 100 °C, and an aliquot was applied to the gel (9). The proteins were stained with Coomassie brilliant blue R-250.

Amino Acid Analysis—Purified protein C23 was hydrolyzed for 22 h \( \text{in vacuo} \) at 110 °C in 6 N HCl. The analyses were performed on a Beckman Model 121 MB amino acid analyzer. Methodology similar to that described by Liu and Chang (10) was used to analyze for the presence of methylated arginines. Acidic and neutral amino acids were eluted from a 25-cm microbore column of AA-10 spherical resin for 50 min with 0.35 N sodium citrate buffer, pH 3.9, at 30 °C. Lysine, histidine, \( \text{N}^\text{V}, \text{N}^\text{VI} \)-dimethylarginine, \( \text{N}^\text{V}, \text{N}^\text{VI} \)-dimethylarginine, and \( \text{N}^\text{VI} \)-monomethylarginine, and arginine were resolved by further elution for 130 min with 0.4 N sodium citrate buffer, pH 5.28, at 50 °C. When the labeled protein C23 hydrolysate was analyzed, 0.5-ml fractions were collected from the analyzer and counted.

RESULTS

Fig. 1 shows one-dimensional polyacrylamide gels of total Novikoff hepatoma nucleolar proteins (Fig. 1A) and purified protein C23 (Fig. 1B). A scan of the gel in Fig. 1A was used to

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Fig. 1. Polyacrylamide gel electrophoresis of total Novikoff hepatoma nucleolar proteins and purified protein C23. A, total nucleolar proteins; B, purified protein C23 (1) on 11% polyacrylamide Laemmli gels (9). All gels were stained with Coomassie brilliant blue. H, histones H2a, H2b, H3, and H4. The molecular weight markers are phosphorylase b (94,000), bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (30,000), and soybean trypsin inhibitor (21,000).
Methylation of Protein C23

TABLE I

Amino acid composition of protein C23

| Amino acid | C23 | C23' | 110,000^a |
|------------|-----|------|-----------|
| Aspartic acid | 11.1 | 12.0 | 10.0 |
| Thr | 5.1 | 5.6 | 5.3 |
| Serine | 5.7 | 5.4 | 8.5 |
| Glutamic acid | 16.7 | 18.5 | 17.5 |
| Proline | 10.6 | 10.0 | 8.9 |
| Glycine | 10.2 | 10.3 | 10.8 |
| Alanine | 5.6 | 6.0 | 5.9 |
| Methionine | 0.9 | 1.2 | 0.9 |
| Isoleucine | 2.9 | 2.5 | 3.1 |
| Leucine | 5.3 | 5.6 | 5.3 |
| Tyrosine | 0.8 | 0.8 | 1.1 |
| Phenylalanine | 3.6 | 3.5 | 3.0 |
| Lysine | 11.1 | 11.8 | 11.0 |
| Histidine | 1.0 | 0.5 | 0.8 |
| Arginine | 2.8 | 2.9 | 3.1 |
| N^\text{O}-N^\text{O}'-Dimethylarginine | 1.3 | ND^b | ND^b |
| N^\text{O}-Monomethylarginine | Trace | ND^b | ND^b |

^a Mamrack et al. (2).
^b Tatsui et al. (6).
^c ND, not determined.

**Fig. 2.** $N^\text{O},N^\text{O}'$-Dimethylarginine in protein C23. Purified protein C23 (1) was hydrolyzed in 6 N HCl for 22 h at 110 °C in vacuo. The hydrolysate was analyzed on a Beckman Model 121 MB amino acid analyzer with a program designed to extend the basic region (see "Materials and Methods"). A, $N^\text{O}$-monomethylarginine; B, $N^\text{O},N^\text{O}'$-dimethylarginine; C, $N^\text{N},N^\text{N}'$-dimethylarginine; D, hydrolysate of protein C23. Approximately 2000 cpm of the hydrolysate of ^14C-labeled protein C23 were loaded on the column; 0.5-ml fractions were collected and counted.

**TABLE II**

$N^\text{O},N^\text{O}'$-Dimethylarginine content in nuclear proteins

| Nuclear protein | $N^\text{O},N^\text{O}'$-d. methylarginine | Ref. |
|----------------|----------------------------------------|-----|
| HeLa A, | 0.1 | 2.9 | 18 |
| HeLa A, | 0.4 | 7.9 | 18 |
| Rat 2, | 0.8 | 13.5 | 16 |
| Rat 6 + 7, | 1.6 | 32.0 | 16 |
| Rat 8, | 1.2 | 17.4 | 16 |
| Rat 10 + 11, | 0.6 | 10.7 | 16 |
| Physarum, | 3.1 | 45.5 | 14 |
| Histones, | P^a | ND^a | ND^a |

^a P, present.
^b The mole per cent of $N^\text{O},N^\text{O}'$-dimethylarginine was calculated from the known arginine concentration in the high mobility groups (19) and from the percentage of arginine as $N^\text{O},N^\text{O}'$-dimethylarginine (13).

**DISCUSSION**

Post-translational methylation of proteins at arginine residues is a common cellular process (11). The enzyme which catalyzes arginine methylation is protein methylase I (S-adenosyl-L-methionine:protein (arginine) N-methyltransferase, EC 2.1.1.23) (12). This enzyme can modify arginine residues to $N^\text{O}$-mon-, $N^\text{O},N^\text{O}'$-, and $N^\text{O},N^\text{O}'$-dimethylarginines. The histones (12), high mobility groups 1 and 2 (19), and heterogeneous nuclear RNA binding proteins (14-16) are nuclear proteins which are known to contain methylarginines. The predominant methylated residue that has been identified in nonhistone chromosomal proteins is $N^\text{O},N^\text{O}'$-dimethylarginine (15, 16). The functional significance of the methylation of nuclear proteins is currently not clear but a direct correlation has been observed between protein methylase I activity levels and the rate of cell proliferation (11).

We report here the presence of $N^\text{N},N^\text{N}'$-dimethylarginine in a high mole per cent (1.3%) in the highly phosphorylated, acidic, ribosomal chromatin-associated protein, protein C23. This protein also contains a trace of $N^\text{N}-monomethylarginine but no $N^\text{O},N^\text{O}'$-dimethylarginine was observed. Approximately one-third of the arginine residues in protein C23 are methylated (Table II). Accordingly, protein C23 is one of the most highly methylated nuclear proteins thus far detected in higher eukaryotes (Table II). Only a few heterogenous nuclear ribonucleoprotein proteins contain a comparable amount of $N^\text{O},N^\text{O}'$-dimethylarginine. Unlike protein C23, these proteins are basic and contain a high mole per cent of glycine. Like protein C23, some are phosphorylated (16). It has been suggested that $N^\text{O},N^\text{O}'$-dimethylarginine may be diagnostic of a
general class of RNA-associated proteins (17). Protein C23 has been found in nucleolar preribosomal particles (18). The functional role of methylation in protein C23 may be similar to that of the methylation of the heterogenous nuclear ribonucleoprotein proteins. However, the presence of $N^\alpha,N^\epsilon$-dimethylarginine in the histones, high mobility groups, and other nonRNA-associated proteins (11) suggests other functional roles exist for this residue.

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