NEGATIVELY CHARGED PHOSPHOPEPTIDES OF NUCLEOLAR NONHISTONE PROTEINS FROM NOVIKOFF HEPATOMA ASCITES CELLS

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SUMMARY

To characterize the sites phosphorylated by endogenous kinases, phosphopeptides of isolated nucleolar nonhistone proteins were analyzed. Major phosphoprotein bands C23 and B23 were ^{32}P labeled in vitro and electrophoretically isolated. Tryptic phosphopeptides were resolved by DEAE-Sephadex chromatography into fractions A, B and C for band C23 and α and β for band B23. Each of these fractions contained phosphoserine, had a distinct amino acid composition of 49-65% glx + asx and 4-11% lys, and had molecular weights of 7-11,000 determined on Sephadex G50. These data indicate that two nucleolar nonhistone proteins have similar phosphorylated regions of high negative charge density.

INTRODUCTION

Phosphorylation of nonhistone nuclear proteins (NHP) has been suggested to participate in the regulation of gene activity (1-5), and in maturation of preribosomal particles (6). Selective phosphorylation of nucleolar acid soluble proteins of Novikoff hepatoma ascites cells was shown to occur in vivo (7,8) and in vitro (9), and two-dimensional polyacrylamide gel electrophoresis resolved approximately 40 ^{32}P labeled nucleolar proteins. Since nucleolus enriched rRNA genes may function by tandem gene control mechanisms requiring common sites of regulation (10), the current studies were initiated to determine if potential regulatory phosphoproteins exhibit common features in their sites of phosphorylation.

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MATERIALS AND METHODS

Nucleoli and Nucleolar Protein Labeling - Novikoff hepatoma ascites cells were transplanted into male albino rats (Holtzman Co., Madison, Wisc.) 6 days prior to the experiments. Nucleoli were isolated as previously described (11). Nucleoli were incubated in 0.25 M sucrose, 5 mM $ZnCl_2$, 12.5 mM NaCl, 0.05 M Tris HCl (pH 7.5) at 37° with three aliquots of 0.1 mCi of γ-^{32}P ATP (20 Ci/mmole) added at ten minute intervals. The mixture was then cooled to 4° and 4 N H_2SO_4 was added to a final concentration of 0.4 N. $ZnCl_2$ was employed to increase isotope incorporation into protein B23 (9).

Protein and Peptide Isolation - The 0.4 N H_2SO_4 soluble proteins were separated by 5% acrylamide acid-urea type preparative slab gel electrophoresis as previously described (12,13). Bands were identified by stained vertical sections or from X-ray films exposed for 8-48 hours. The protein bands were electrophoresed through 5% polyacrylamide gel plugs into dialysis bags for 30 hours at 120 volts (constant voltage). The proteins were dialyzed against 0.05 M acetic acid and then digested with trypsin for 4 hrs at 37° in 0.1 M N-ethyl morpholine-acetate, pH 7.5.

The tryptic peptides were separated by DEAE-Sephadex A-25 columns (0.7 x 25 cm) with a running buffer of 7 M urea, 0.05 M Tris pH 7.5 and a linear gradient of 0.1 M to 0.6 M NaCl. Oligodeoxythymidine markers (Collaborative Research, Inc., Waltham, Mass.) were used to approximate the net negative charge. Fractions were counted by the Cerenkov method (14).

RESULTS

The 0.4 N H_2SO_4 soluble nucleolar proteins labeled *in vitro* with γ-^{32}P ATP were separated by preparative acid-urea slab gels. The autoradiogram in Figure 1 indicates that the major ^{32}P labeled bands correspond to bands C23 and B23 on the stained gel pattern (7,15). When these bands were further analyzed by 8% polyacrylamide gels containing SDS (15), over 95% and 87% of the radioactivity was in the major bands C23 and B23, respectively.

The tryptic digests were separated under conditions suitable for charge dependent separation of oligonucleotides (16). The direct application of the total digests of *in vitro* labeled proteins resulted in the patterns shown in Figure 2. Three phosphopeptide fractions (A, B and C) were obtained from protein band C23; fraction C had the highest net negative charge eluting at approximately 0.45 M NaCl. Band B23 phosphopeptides separated

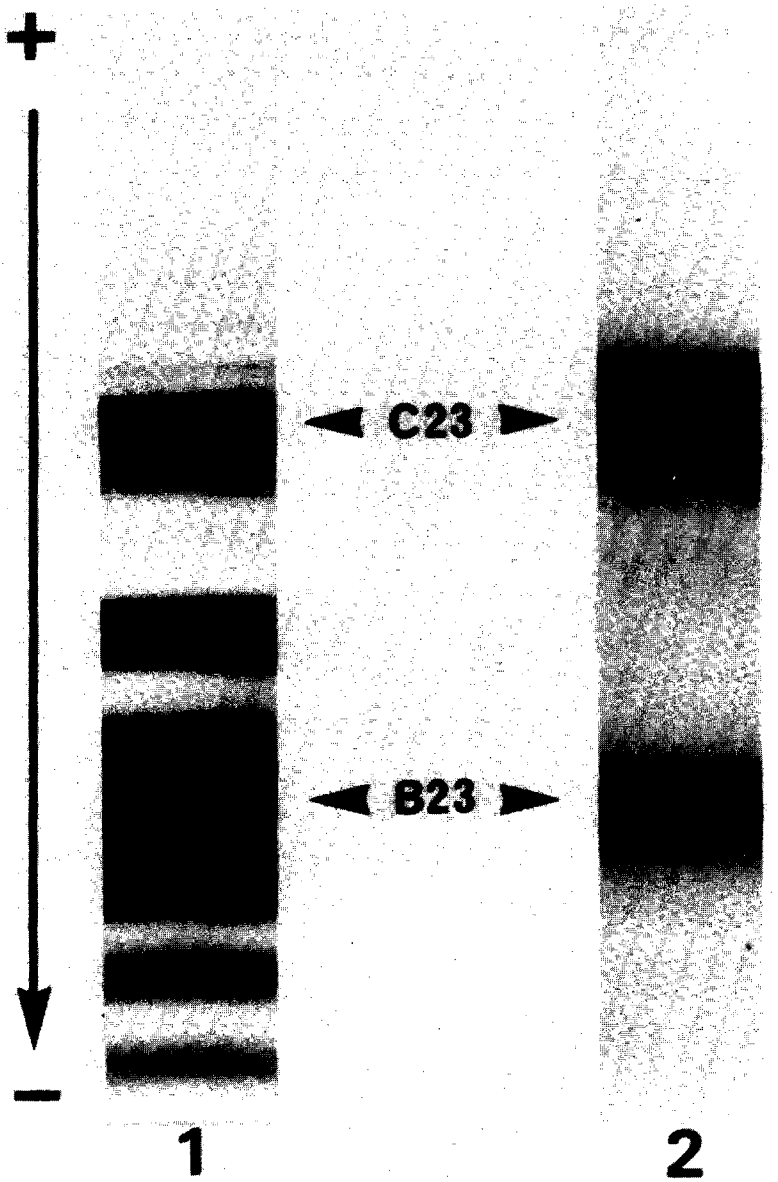

Figure 1 Separation of *in vitro* ^{32}P labeled phosphoproteins of nucleolar acid extract. Preparative 5% acrylamide acid-urea slab gels (8 x 9.5 cm) were loaded with 10 mg protein per slab and run at 120 volts for 3.5 hours. (1) Portion of slab gel stained in 1% amido black, 7% acetic acid. (2) Portion of autoradiograph exposed for 24 hours. Major phosphoproteins C23 and B23 are denoted by arrows.

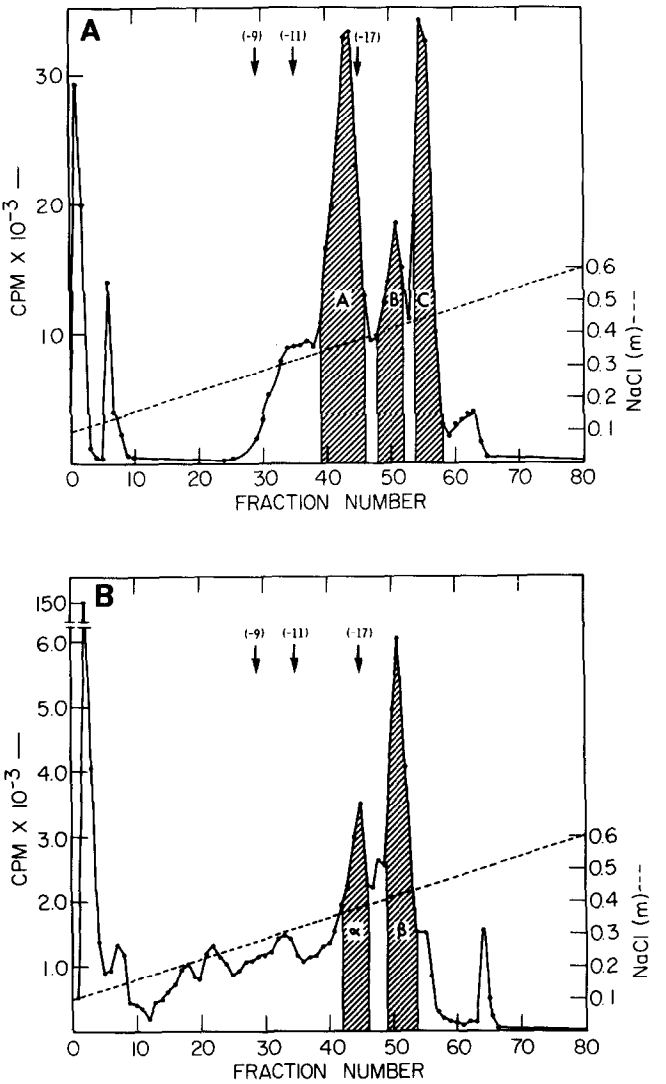

Figure 2 Separation of tryptic phosphopeptides on DEAE-Sephadex. (A) Tryptic digest of protein band C23 was separated in 7 M urea, 0.05 M Tris pH 7.5 with a 0.1 M to 0.6 M NaCl gradient. Oligodeoxythymine markers were used to estimate the charge (arrows at top). (B) Tryptic digest of protein band B23 treated as in (A).

into two major peaks, α and β (Fig. 2b). Phosphopeptide fractions C23-B, C23-C and B23-B had net negative charges greater than seventeen relative to oligo dT markers.

The amino acid composition of the peaks are shown in Table

TABLE I

AMINO ACID COMPOSITION OF PHOSPHOPEPTIDE FRACTIONS CORRECTED MOLE PERCENTS[a]

	Band C23			Band B23	
	A	B	C	α	β
Lys	8.5	8.5	4.2	6.0	10.8
His	0	0	0	2.3	0.1
Arg	0	0	0	0.9	0.2
Asx	16.0	30.6	25.6	22.6	35.7
Thr[b]	0.9	0.9	2.5	1.7	3.2
Ser[c]	9.2	5.6	5.3	7.4	1.3
Glx	33.6	34.6	31.0	26.3	29.2
Pro	9.7	5.6	7.3	3.0	2.7
Gly	4.8	2.7	0.9	7.9	1.5
Ala	4.0	4.0	13.0	6.5	1.0
Val	6.2	4.5	3.7	7.0	8.1
Met	3.5	0.3	1.1	0.5	0
Ileu	2.0	0.3	2.3	1.2	0
Leu	0.6	0.3	0.1	4.7	3.4
Tyr	0	0	0	0	0
Phe	0	2.1	0.6	0.3	3.0
Pser[d]	3.0	1.3	1.4	1.8	0.2
Asx,Glx/ Lys,His, Arg	5.8	7.7	13.5	5.3	6.0
M.W.[e]	11,000	10,000	11,000	7,000	10,000

a) Peptides were hydrolyzed in 5.7 N HCl at 110°C for 22 hrs in tubes flushed with nitrogen and sealed in vacuo. For phosphoserine (Pser) and phosphothreonine (Pthr) analysis, the hydrolysis was for 8 hours at 110° in 2 N HCl. Each composition is an average of at least two preparations.

I. Each tryptic peptide fraction has a distinct composition including 4-11 mole% lysine and 0.2 to 3.0 mole% phosphoserine with no detectable phosphothreonine. The striking feature of the amino acid compositions is the high content of acidic residues (49-65 mole% glx + asx). The molecular weights of each peptide fraction were estimated to be 7,000-11,000 using a Sephadex G-50 fine column under denaturing conditions (1.0 M NaCl, 5 M urea, 0.05 M Tris, pH 7.5).

DISCUSSION

The two nucleolar nonhistone proteins C23 and B23 have been partially purified and shown to contain regions of similarity at the phosphorylated sites. Large highly negatively charged phosphopeptides separated by DEAE-Sephadex were found in the two protein fractions. These peptide fractions have similar but distinct amino acid compositions; each peptide is composed of at least 49 mole percent acidic residues in addition to phosphoserine. Since the maximum number of phosphoserine residues per molecule is three (Table I), the majority of the net negative charge is contributed by the acidic residues.

Each peptide contains lysine residues which are insensitive

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b) The value for threonine includes correction for 7% destruction during hydrolysis.

c) The value for serine includes correction for 10% destruction during hydrolysis.

d) The value for phosphoserine includes correction for 70% destruction during hydrolysis determined by standards hydrolyzed in parallel.

e) Estimation of molecular weight by Sephadex G50 chromatography in which peptide fractions from the DEAE-Sephadex chromatography (Fig. 2) were pooled, desalted and redissolved in 1.0 M NaCl, 5.0 M urea, 0.01 M Tris, pH 7.5 and applied to a 1.5 x 40 cm column of Sephadex G50 fine equilibrated with the same buffer. RNase A, insulin and bacitracin were used as molecular weight markers.

to trypsin. There are several possible explanations for this trypsin insensitivity. Lysine residues next to phosphorylated serine or threonine were resistant to trypsin in three phosphopeptides of the H1 histone (17). Alternatively, histone H2A contained two adjacent lysine residues which conferred trypsin insensitivity to one of them (18). A third possibility could be the presence of chemically modified lysine residues (e.g. acetylation).

The nucleolar acid extract contains many proteins including histones (7) and proteins associated with preribosomal particles (19). In preparations of preribosomal particles, spot C23 is phosphorylated whereas spot B23 is not (6). These proteins may be involved in the regulation of ribosomal RNA (rRNA) transcription or in rRNA processing and preribosomal particle formation. It is possible that their functions are coordinated by site specific kinases and phosphatases as with the phosphorylation of tissue specific proteins by cytoplasmic cAMP dependent protein kinase in response to hormones and drugs (20,21).

An intriguing question is what role does the addition of phosphate residues to an already highly negatively charged region of the protein play in the function of these proteins. Are these regions of the proteins involved in unique interactions with nucleic acids or with histones? Such questions can only be answered when the structures and functions of these polypeptides are more precisely defined.

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