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A SURVEY OF PEPTIDES CONTAINING SITES OF PHOSPHORYLATION IN NONHISTONE NUCLEAR PROTEINS

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SUMMARY

^{32}P-labeled peptides obtained by pronase digestion of unfractionated nonhistone nuclear proteins were resolved on columns of Dowex 50, DEAE-Sephadex, Bio-Gel P2, and paper electrophoresis at pH 1.8. Each of 30 peptides analyzed contained predominantly glycine, glutamic acid and proline. The chain length ranged from 7 to 19 residues, including 1 to 4 phosphorylated residues per peptide. These results suggest phosphorylation sites in nonhistones involve a high negative charge density in non-helical regions of these proteins.

INTRODUCTION

The structural nature of protein phosphorylation sites has been of increased interest in recent years following the demonstration that the primary sequences of amino acids surrounding these sites are involved in the specificity of kinases (1,2). These sequences have been determined in most of the histones and in numerous cytoplasmic proteins (3). However, relatively little is known about the nature of these sites in the highly phosphorylated nonhistone proteins of the cell nucleus.

Recently, Mamrack et al (4,5) found phosphorylation sites within clusters of acidic amino acids in two nonhistone nucleolar proteins. To determine whether this was a general phenomenon, studies were initiated to survey the sites of phosphorylation in a crude mixture of nonhistone chromatin proteins.

METHODS AND MATERIALS

Acid-insoluble nonhistone nuclear chromatin proteins, consisting of at least 16 major individual phosphoproteins, were

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prepared from Novikoff hepatoma ascites cells labeled *in vitro* with ^{32}P-orthophosphate as previously described (6). Residual nucleic acid contamination was eliminated by treating the proteins in 10% trichloroacetic acid for 20 minutes at 90°C. The proteins were dialyzed against 0.05 N acetic acid, made 0.1 N in N-ethyl morpholine (pH 7.5), and digested with pronase (Calbiochem, La Jolla, Calif.) at a concentration of 50 µg/ml for 18 hours at 37°. The soluble material was flash evaporated, redissolved in 0.05 N HCl and applied batchwise to Dowex 50X8 resin equilibrated in 0.05 N HCl. The resin was poured into a column and eluted with a 0.01-2 M linear gradient of pyridine-acetic acid buffer, pH 4.5 (50 ml of each solution).

Fractions from the Dowex-50 column were flash evaporated and redissolved in 7 M urea, 0.05 M Tris (pH 7.5). Phosphopeptide fractions were applied to a DEAE-Sephadex A25 column and eluted with a 0-0.4 M NaCl linear gradient (400 ml of each buffer) containing 7 M urea and 0.05 M Tris (pH 7.5) To estimate the net negative charge, oligoriboadenylate markers (A_3,A_5, and A_7) having no terminal phosphates (Collaborative Research, Waltham, Mass.) were run under the same conditions (4).

DEAE-Sephadex phosphopeptide fractions were further fractionated by Bio-Gel P-2 column chromatography in a buffer containing 2% formic acid, 8% acetic acid (pH 1.8). Fractions corresponding to peaks of radioactivity were subjected to paper electrophoresis at pH 1.8 (3000 V, 45 min.). Phosphopeptides, localized by autoradiography, were eluted with water.

Isolated phosphopeptides were hydrolyzed in 5.7 N HCl for 22 hours *in vacuo* at 110°C and applied to a Beckman automatic amino acid analyzer. Calculation of actual residue numbers from the amino acid analyses, including the number of phosphorylated residues, was accomplished by assuming the net charge at pH 1.8 (estimated by paper electrophoresis) was determined by the free amino terminus (+1 charge), the basic residues (+1 each) and the phosphorylated residues (negative charge <1) (6) since carboxyl groups are not ionized at this pH.

RESULTS

When ^{32}P-labeled phosphopeptides obtained from pronase digests of acid-insoluble nonhistone nuclear proteins of Novikoff hepatoma were eluted from a Dowex-50 column, a single peak of radioactivity was obtained (Fig. 1a). A small peak eluting in the void volume represented inorganic phosphate (7) released during the incubation with pronase. The phosphopeptide peak was fractionated into nine peaks by DEAE-Sephadex column chromatography (Fig. 1b). Each of the DEAE-Sephadex fractions was separated into as many as four peaks by Bio-Gel P-2 column chromatography (Fig. 2).

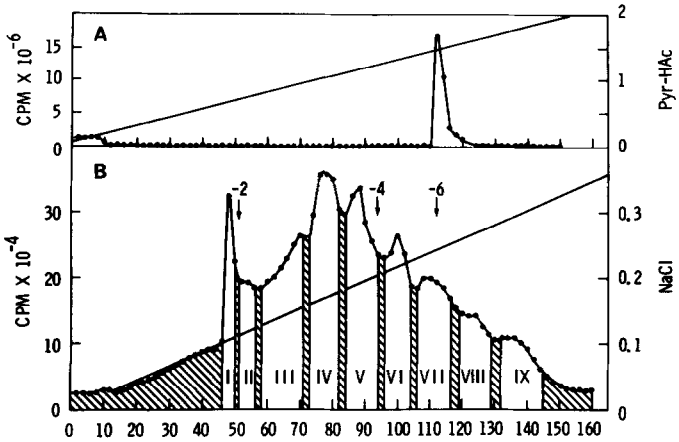

Fig. 1. A) Column profile of ^{32}P-labeled pronase generated phosphopeptides on a Dowex-50 column. Elution was with a 0 to 2 N pyridine-acetic acid gradient, pH 4.5. B) Column profile of peak from Dowex-50 column fractionated by DEAE-Sephadex column chromatography. Elution was with a 0 to 0.4 M NaCl linear gradient containing 50 mM Tris, pH 7.5 and 7 M urea.

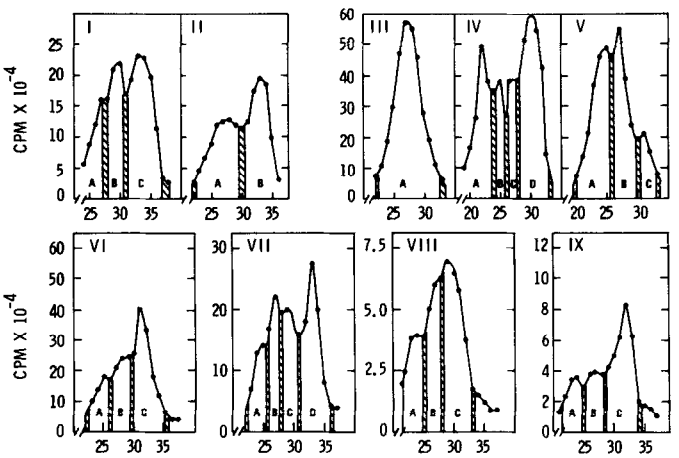

Fig. 2. Column profiles of fractions from the DEAE-Sephadex column (Fig. 1B) upon Bio-Gel column chromatography.

All of the fractions were subjected to pH 1.8 paper electrophoresis and radioactive spots were localized by autoradiography (Fig. 3). No significant quantities of radioactivity migrated off the paper as noted in experiments employing shorter electrophoresis times. The peptides fell into three groups according to electrophoretic mobility. Peptides in Group I (peptides 1-7,

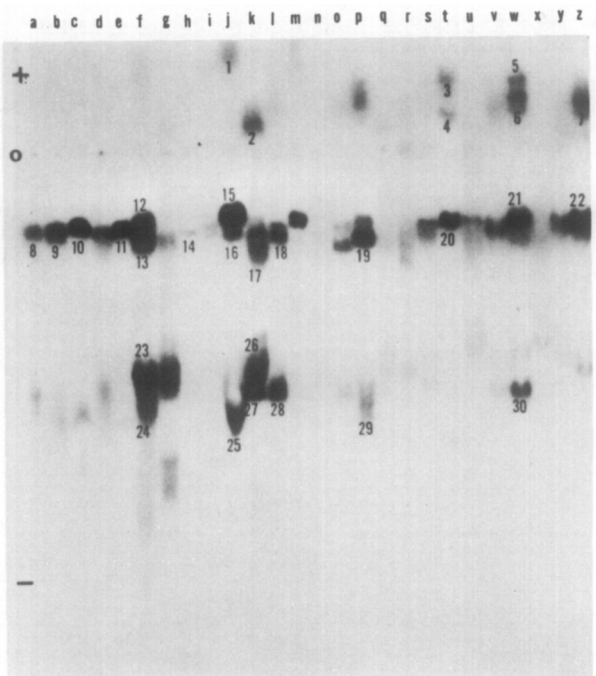

Fig. 3. Autoradiograph of pH 1.8 paper electrophoretogram of fractions from Bio-Gel P-2 columns (Fig. 2). Samples a) through z) correspond to fractions IA through IXC in Figure 2. Peptides 1-7 correspond to Group I, peptides 8-22 correspond to Group II, and peptides 23-30 correspond to Group III in the text.

Fig. 3) were negatively charged and moved a short distance (1-5 cm) off the origin toward the positive pole. Since at pH 1.8 each phosphate contributes less than one full negative charge unit (6), these peptides must contain two more phosphate residues than basic residues (the N-terminal amino group contributes one full positive charge unit). Group II peptides (8-22), which were positively charged, also migrated a short distance (5-6 cm) from the origin. Therefore, they contained phosphoryl groups exceeding basic residues by one. Group III peptides (23-30) were also positively charged but migrated more than twice as rapidly as the Group II peptides. In this group, the number of phos-

TABLE I

Amino Acid Ratios of Isolated Phosphopeptides[a]

Peptide Number	1	4	6	8	12	16	18	19	20	27
Asx	0.7	0.9	2.2		0.9	0.9	0.6	0.9		1.1
Thr[b]	1.3		1.0	0.9		0.4		0.4	0.6	
Ser[b]	2.8	1.8	2.7	2.0	1.5	1.3	1.4	1.3	1.0	1.2
Glx	1.1	1.1	0.8	1.0	1.3	1.2	0.8	1.1	2.8	1.9
Pro	5.9	1.8	1.8	4.2	3.0	1.1	1.0	0.9	1.6	1.2
Gly	2.2	1.2	2.9	2.0	2.0	1.0	0.9	0.7	1.7	1.2
Ala	1.1		1.0	0.8	0.8	0.7	0.3	0.2		0.8
½Cys						0.9				
Val								0.2		0.6
Tyr		0.8								
Lys	1.0			1.1		0.9		0.3		0.8
His			0.8							
Arg	1.1	1.2	1.0	0.8		2.0	0.9	1.8		0.7
PSer+PThr[c]	4	3	4	3	1	4	2	3	1	2
Approximate Net Charge (pH 7.5)[d]	-3	-5	-6	-2	-3	-3	-4	-4	-6	-4

a. Tryptophan was not determined. Met, Ile, Leu and Phe were not detected in these peptides.

b. Threonine and serine values are uncorrected. The values represent the sum derived from phosphoamino acid breakdown (50-80% yield (6)) plus any nonphosphorylated speicies.

c. The sum of phosphoserine and phosphothreonine was calculated from the mobility at pH 1.8 and the number of basic residues (see text).

d. The net charge at pH 7.5 was calculated from the position of elution from the DEAE-Sephadex column (Figure 1).

phorylated residues must equal or be less than the number of basic residues to produce a positive charge of greater than one.

The amino acid ratios for ten peptides representative of 30 studied are presented in Table I. Table I also presents the approximate net charge of the peptides at pH 7.5, which was derived from DEAE-Sephadex elution data (Fig. 1b).

A number of striking features regarding the amino acid compositions of these peptides were observed. Three amino acids (glycine, glutamic acid and proline) were common to all phosphopeptides analyzed. Arginine and aspartic acid were also relatively abundant. Hydrophobic amino acids were rarely found. Methionine was not found at all. Most of the peptides analyzed appeared to contain multiple phosphorylation sites in a single small peptide.

Estimations of the observed net negative charges of these peptides at pH 7.5 (based on elution from the DEAE-Sephadex column (4) and the expected values (assuming the charge contributed by the phosphate to be about minus 1.35 (9) and assuming integral values for other functional groups) were in good agreement for most peptides (calculations not shown). This suggested that few of these peptides contained asparagine or glutamine. However, it cannot be ruled out that deamidation did not take place during the brief TCA hydrolysis procedure used to remove contaminating nucleic acids.

DISCUSSION

This study suggests that in nonhistone proteins the amino acids which are most abundant near the sites of phosphorylation are proline, glutamic acid and glycine. The prevalence of proline in these phosphopeptides suggests a lack of helical secondary structure is required in these regions either in relation to kinase and phosphatase recognition or in relation to the function of these regions.

The abundance of glutamic acid near phosphorylation sites was perhaps not unexpected since nuclear protein kinases act effectively on casein (9) which contains glutamic acid residues near phosphorylatable amino acids (1). The prevalence as well

of other negatively charged side chains (aspartic acid and the apparent clustering of up to four phosphoamino acids in a single pronase generated phosphopeptide) suggests that phosphorylation and dephosphorylation provide a mechanism for producing and regulating a "high negative charge density" (10) in one or more regions of a nonhistone nuclear protein. This suggests that these regions might be involved in electrostatic interactions with positively charged regions of histones. The results of McCleary et al (11) have, in fact, already suggested that phosphorylation of nonhistones is associated with the binding of these proteins to histones.

Despite the abundance of acidic residues, the presence of other amino acids in these peptides indicates these phosphorylation sites differ somewhat from those found in two nucleolar proteins which contain long stretches of almost exclusively acidic residues (4,5).

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