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Journal of Chromatography A, 782 (1997) 55–62

JOURNAL OF CHROMATOGRAPHY A

Application of hydrophilic-interaction liquid chromatography to the separation of phosphorylated H1 histones

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Received 5 February 1997; revised 9 April 1997; accepted 22 April 1997

Abstract

A new two-step high-performance liquid chromatography (HPLC) procedure has been developed to separate modified histone H1 subtypes. Reversed-phase (RP) HPLC followed by hydrophilic-interaction liquid chromatography (HILIC) was used for analytical and semi-preparative scale fractionation of multi-phosphorylated H1 histone subtypes into their non-phosphorylated and distinct phosphorylated forms. The HILIC system utilizes the weak cation-exchange column PolyCAT A and an increasing sodium perchlorate gradient in a methanephosphonic acid–triethylamine buffer (pH 3.0) in the presence of 70% (v/v) acetonitrile. The identity and purity of the individual histone subfractions obtained was assayed by capillary electrophoretic analysis. The results demonstrate that application of the combined RP-HPLC–HILIC procedure to the analysis and isolation of modified H1 histone subtypes provides an innovative and important alternative to traditional separation techniques that will be extremely useful in studying the biological function of histone phosphorylation. © 1997 Elsevier Science B.V.

Keywords: Hydrophilic-interaction liquid chromatography; Histones; Proteins

1. Introduction

Histones are a family of proteins that participate in organizing the structure of eukaryotic chromatin, and the reversible chemical modifications of acetylation, phosphorylation and ubiquitination of these nuclear proteins are associated with dynamic changes in chromosome packaging during the cell cycle (for a review, see Ref. [1]). The H1 histones which bind to the outside of the nucleosomes are a heterogeneous group of several subtypes differing in their primary sequences [2,3]. Relative proportions vary according to tissue and species and change during development and differentiation [2,4–6]. Histone H1 subtypes differ in their ability to condense DNA, which means it is possible that the differential distribution of the H1s with chromatin domains may cause differential compaction of chromatin [2,5]. Since it is thought that chromatin structure plays an important role in regulating transcription by controlling the accessibility of DNA to transcription factors [7–9], histone H1 molecules have received considerable attention. Studies of recent date also suggest that certain antirepressors and antirepressor activities of some transcription factors interact specifically with H1 histones [10–12].

In addition to the heterogeneity of their primary sequences, H1 histones are also post-translationally modified. The H1 histones are phosphorylated at serine and threonine residues located in the N- and

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PII S0021-9673(97)00468-8

C-terminal domains of the protein [13]. H1 phosphorylation is enhanced in exponentially growing cells and diminished in non-proliferating or quiescent cells. During the cell cycle, phosphorylation begins in the late G1 phase, increases throughout the S and G2 phases and reaches a maximum in mitosis. In addition, individual H1 subtypes differ in their extent of phosphorylation [14–17]. Phosphorylation of the H1 subtypes is thought to influence their interaction with DNA and this could affect modulation of chromatin structure [18,19]. On the one hand, it has been proposed that H1 phosphorylation triggers chromosome condensation during mitosis [1]. On the other, however, it has also been suggested that phosphorylation of H1 acts as a first-step mechanism in inducing chromatin decondensation, enabling access of factors for gene activation or replication as well as chromosome condensation [19]. The exact biological function of histone H1 phosphorylation is still unknown.

In order to study chromatin structure/function relationships of histone phosphorylation it is essential to perform reconstitution studies of chromatin with precisely defined phosphorylated H1 histones and DNA sequences. This requires a fast and efficient method for the preparative-scale isolation of differently phosphorylated H1 histone subtypes. In the past many attempts have been made to resolve all histone H1 subtypes and their phosphorylated forms. Separations were achieved by virtue of two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE) [20] or by using BioRex 70 column chromatography in combination with 2D-PAGE [16]. These labour-intensive and extremely time-consuming methods were, however, exclusively suited for analytical applications. Subsequently, a particularly fast analytical high-performace capillary electrophoresis (HPCE) method allowing excellent resolution of non-phosphorylated and various phosphorylated H1 histone variants was described by Lindner et al. [21].

A chromatographic technique utilizing polar sorbents with hydrophobic mobile phases has proven to be an efficient method for separating polar species including amino acids, peptides and carbohydrates [22–24]. The efficiency of this so-called hydrophilic-interaction liquid chromatography (HILIC) was recently demonstrated by applying it to the analytical and semi-preparative scale isolation of histone H2A variants, acetylated H2A variants and acetylated H4 histones [25].

This paper reports on the development of a fast and sensitive HILIC method for separating phosphorylated H1 histone variants on both a semi-preparative and an analytical scale.

2. Materials and methods

2.1. Chemicals

Sodium perchlorate ($NaClO_4$) and triethylamine (TEA) were purchased from Fluka (Buchs, Switzerland), hydroxypropylmethyl cellulose (HPMC; 4000 cP) and trifluoroacetic acid (TFA) were obtained from Sigma (Munich, Germany) and ethylene glycol monomethyl ether (EGME) was ordered from Aldrich (Sternheim, Germany). All other chemicals were purchased from Merck (Darmstadt, Germany).

2.2. Cell line and culture conditions

Raji cells, originally derived from patients with Burkitt's lymphoma, were cultured in RPMI-1640 medium (Biochrom, Berlin, Germany) supplemented with 10% (v/v) fetal calf serum (FCS) (Sebak, Suben, Austria), penicillin (60 μg/ml) and streptomycin (100 μg/ml) in the presence of 5% CO_2. Exponentially growing cells were harvested at a density of $8–9 \cdot 10^5$ cells/ml.

2.3. Sample preparation

Raji cells ($6–7 \cdot 10^9$) were collected by centrifugation (800 *g* for 10 min). The cells were washed with buffer A (0.05 *M* Tris, pH 7.5; 0.025 *M* KCl, 0.01 *M* $CaCl_2$, 0.01 *M* $MgCl_2$, 0.25 *M* sucrose, 0.01 *M* 2-mercaptoethanol, 0.001 *M* phenylmethylsulfonyl fluoride, 0.05 *M* $NaHSO_3$). After centrifugation (800 *g* for 10 min) the cells were homogenized in buffer A containing 0.1% Triton X-100 in a Dounce homogenizer. Nuclei thus obtained were collected by centrifugation (900 *g* for 10 min), and the nuclear pellet was washed once with buffer A without Triton X-100. The pellet was centrifuged down and thereupon extracted with 5% (w/v) $HClO_4$ containing

0.05 *M* $NaHSO_3$ for 60 min with occasional vortex-mixing. $HClO_4$-insoluble material was removed by centrifugation at 12 000 *g* for 10 min. The pellet obtained was re-extracted (30 min) with 5% (w/v) $HClO_4$ containing 0.05 *M* $NaHSO_3$ and re-centrifuged. TCA to a final concentration of 20% (v/v) in the presence of protamine sulphate (20 μg/ml) was added to the combined supernatants. After 60 min the H1 histones were centrifuged down (12 000 *g* for 10 min), washed twice with cold acidified acetone and three times with pure acetone, dissolved in water containing 0.01 *M* 2-mercaptoethanol and lyophilized.

2.4. High-performance liquid chromatography

The equipment used consisted of two 114M pumps, a 421A system controller and a Model 165 variable-wavelength UV–Vis detector (Beckman Instruments, Palo Alto, CA, USA). The effluent was monitored at 210 nm and the peaks were recorded using Beckman System Gold software.

TEA–methanephosphonic acid (MPA) buffers were prepared by adding appropriate amounts of a 1 *M* stock solution prepared by adding TEA to MPA until a pH of 3.0 was reached. Buffer compositions are expressed as (v/v) throughout this text.

2.5. Reversed-phase HPLC

The separation of whole linker histones from logarithmically growing Raji cells was performed on a Nucleosil 300-5 C_4 column (250 mm×8 mm I.D.; 5 μm particle pore size; 30 nm pore size; end-capped; Macherey-Nagel, Düren, Germany). The lyophilized proteins were dissolved in water containing 0.2 *M* 2-mercaptoethanol, and samples of 200 μg were injected onto the column. The histone H1 sample was chromatographed within 35 min at a constant flow of 1.5 ml/min with a acetonitrile gradient starting at solvent A–solvent B (53:47) (solvent A: water containing 0.1% TFA; solvent B: 70% acetonitrile and 0.1% TFA). The concentration of solvent B was increased linearly from 47% to 55% within 35 min, the histone H1.1 fraction collected and, after adding protamine sulphate (20 μg/ml) and 0.01 *M* 2-mercaptoethanol, lyophilized and stored at −20°C.

2.6. Hydrophilic-interaction liquid chromatography

The histone fraction H1.1 (20–150 μg) isolated by RP-HPLC was analysed on a PolyCAT A column (200 mm×4.6 mm I.D.; 5 μm particle pore size; 30 nm pore size; ICT, Vienna, Austria) at 20°C and at a constant flow of 1.0 ml/min using a two-step gradient starting from solvent A–solvent B (100:0) (solvent A: 70% acetonitrile, 0.01 *M* TEA–MPA, pH 3.0; solvent B: 70% acetonitrile, 0.01 *M* TEA–MPA, pH 3.0 and 1 *M* $NaClO_4$). The concentration of solvent B was increased from 0 to 60% B during 5 min and from 60% to 100% B during 30 min. In order to fractionate all the histone H1.1 sample (up to 500 μg) the concentration of solvent B was increased from 0% to 60% during 5 min and from 60% to 100% during 60 min. The isolated protein fractions were desalted using RP-HPLC. Histone fractions obtained in this way were collected and after adding protamine sulphate (20 μg/ml) and 0.01 *M* 2-mercaptoethanol lyophilized and stored at −20°C.

2.7. Cation-exchange chromatography

The histone fraction H1.1 isolated by RP-HPLC was analysed on the same PolyCAT A column used for HILIC. At 20°C and at a constant flow of 1.0 ml/min a two-step gradient was applied starting from solvent A–solvent B (100:0) (solvent A: 0.01 *M* sodium phosphate, pH 3.0, solvent B: 0.01 *M* sodium phosphate and 1 *M* $NaClO_4$) to 40% B (5 min) and from 40% to 70% B (30 min).

The separation of the histone fraction H1.1 in the presence of 40% acetonitrile was performed using a two-step gradient starting from solvent A–solvent B (100:0) (solvent A: 40% acetonitrile, 0.01 *M* TEA–MPA, pH 3.0; solvent B: 40% acetonitrile, 0.01 *M* TEA–MPA and 1 *M* $NaClO_4$) to 15% B (5 min) and from 15% to 45% B (30 min).

The isolated protein fractions were desalted using RP-HPLC. Histone fractions obtained in this way were collected and, after adding protamine sulphate (20 μg/ml) and 0.01 *M* 2-mercaptoethanol, lyophilized and stored at −20°C.

2.8. Capillary electrophoresis

High-performance capillary electrophoresis (HPCE) was performed on a Beckman system P/ACE 2100 controlled by an AT486 computer. Data collection and post-run data analysis were carried out using P/ACE and System Gold software (Beckman Instruments). The capillary cartridge used was fused-silica 67 cm (60cm to the detector)×75 μm I.D.. Protein samples were injected by pressure and detection was performed by measuring UV absorption at 200 nm. In all experiments an untreated capillary was used. Runs were carried out in 0.1 *M* phosphate buffer (pH 2.0) containing 0.02% HPMC at a constant voltage (12 kV) and at a capillary temperature of 25°C.

2.9. Incubation of histones H1 with alkaline phosphatase

About 100 μg of whole histones in 0.25 ml of 0.01 *M* Tris buffer (pH 8.0) and 0.001 *M* phenylmethylsulphonyl fluoride (PMSF) were mixed with 210 μg of *E. coli* alkaline phosphatase (60 units/mg; Sigma) for 12 h at 37°C according to Sherod et al. [26].

3. Results and discussion

It is generally known that no chromatographic technique permits the simultaneous separation of all histones and their modified forms [27–37]. We have previously shown, for example, that separating post-translationally acetylated core histones and core histone variants requires a combined RP-HPLC–HILIC system [25]. In this study we were interested in developing a chromatographic procedure for separating phosphorylated H1 histones. Since the degree of histone H1 phosphorylation is generally higher in proliferating than in quiescent or non-dividing cells [38], we isolated whole linker histones from exponentially growing cultures of Raji cells, originally derived from patients with Burkitt's lymphoma, and pre-separated the proteins using RP-HPLC with a semi-preparative column filled with Nucleosil 300-5 C_4 and a multi-step water–acetonitrile gradient. We obtained just two subfractions (Fig. 1), which were characterized by sodium dodecylsulfate (SDS) and acid/urea/triton (AUT) PAGE (data not shown). In this context it should be noted that Raji cells do not synthesize histone H1° [39]. The first subfraction (designated H1.1), which eluted between 17 min and 19 min, corresponds to the human "H1.5" in the nomenclature of Eick et al. [40] and Albig and coworkers [41–43] and the murine "H1b" in the nomenclature of Lennox et al. [20] and has been proven to be pure and not contaminated by any other protein (data not shown). The second peak (designated H1.2), however, consisted of a mixture of several histone H1 subtypes. Applying an HPCE method (uncoated capillary, 0.1 *M* phosphate buffer with pH 2.0 containing 0.02% HPMC) used for the resolution of multi-phosphorylated H1 variants of NIH 3T3 cells [18], the histone H1.1 subfraction, which was a single peak in RP-HPLC, was further separated into four peaks belonging to the non- (designated $H1.1p_0$), mono- ($H1.1p_1$), di- ($H1.1p_2$) and tri-phosphorylated ($H1.1p_3$) forms of histone H1.1 (shown in Fig. 2a). To prove the existence of highly phosphorylated forms, the histone H1.1 subfraction used for the HPCE analysis shown in Fig. 2a was digested using alkaline phosphatase. After treatment with the enzyme, the sample was chromatographed (data not shown) and subjected to HPCE (Fig. 2b). In contrast to Fig. 2a, only two peaks were detectable: one prominent peak consisting of non-phosphorylated

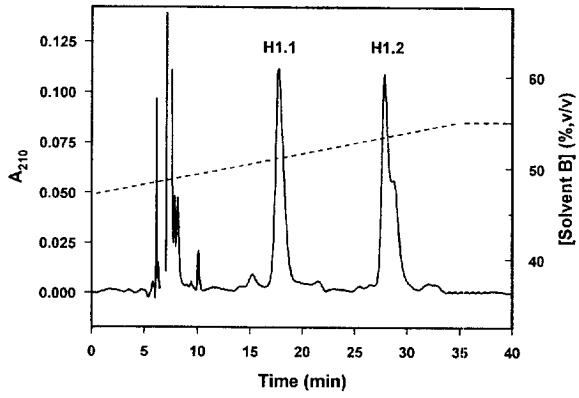

Fig. 1. Separation of whole linker histones by RP-HPLC. Amounts of 200 μg of H1 histone sample isolated from exponentially growing Raji cells were injected onto a Nucleosil 300-5 C_4 column as described in Sections 2.2, 2.3 and 2.5.

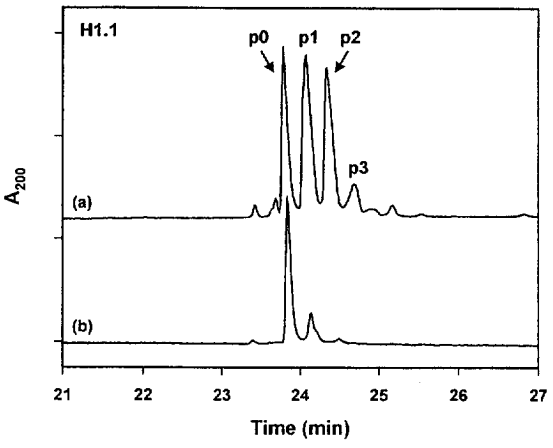

Fig. 2. HPCE of phosphorylated histone H1.1 before and after digestion with alkaline phosphatase. CE conditions were as follows: 0.1 *M* sodium phosphate buffer, pH 2.0, with 0.02% HPMC; injection time 3 s; voltage applied 12 kV; temperature constant at 25°C; detection at 200 nm; untreated capillary; 60 cm×75 μm; (a) HPCE of the histone H1.1 fraction isolated by RP-HPLC (Fig. 1); (b) the histone H1.1 fraction isolated by RP-HPLC (Fig. 1) was digested with alkaline phosphatase (see Section 2.8) and subjected to HPCE; p_0, p_1, p_2, p_3=non-, mono-, di-, tri-phosphorylated H1.1 histones.

histone H1.1 and one minor peak of mono-phosphorylated H1.1. Therefore, the total loss of the higher phosphorylated forms of H1.1 ($H1.1p_2$ and $H1.1p_3$) and the clear decrease in $H1.1p_1$ confirm our assignment of peaks in Fig. 2a.

In the past, the fractionation and purification of phosphorylated H1 subcomponents has generally relied on conventional ion-exchange chromatography (IEC) using Bio-Rex 70 with a very shallow guanidinium chloride gradient. This technique, however, normally requires extensive separation time and does not permit isolation of pure distinct phosphorylated forms [14]. Recently, Lu et al. [44] have developed a more rapid chromatographic method. By applying cation-exchange HPLC, phosphorylated forms of tetrahymena macronuclear histone H1 were separated from each other and from dephosphorylated H1. We were interested, therefore, in testing the HPLC method described for the separation of phosphorylated mammalian H1 histones. When the histone H1.1 subfraction obtained from human Raji cells at pH 6.5 was separated with a linear gradient of increasing sodium perchlorate concentration (0–2 *M*) using the weak cation-exchange column PolyCAT A, three peaks were observed. However, only the tri-phosphorylated histone H1.1 was clearly separated and isolated in a pure form, whereas both the non- and di-phosphorylated proteins were contaminated by the mono-phosphorylated derivative (data not shown). As would be expected, the histone protein separation mechanism was electrostatic in nature with the more highly positive charged $H1.1p_0$ being eluted later than the lesser charged $H1.1p_1$, $H1.1p_2$ and $H1.1p_3$. When the histone H1.1 subfraction was fractionated under the same run conditions, but at pH 3.0 instead of pH 6.5, only two peaks were obtained (demonstrated in Fig. 3). The shoulder contained tri-phosphorylated histone H1.1, whereas the two peaks consisted of a mixture of histones as described above. Furthermore, it should be noted that in the IEC mode we have found remarkable differences in the resolving power for phosphorylated H1 histones depending on the PolyCAT A column used. In some cases, for example, only a single broad peak was achieved (data not shown). Summarizing, ion-exchange HPLC has proven to be an unsuitable technique for separating phosphorylated human H1 subtypes. In this context it should be pointed out that phosphorylation of specific serine- and/or threonine residues reduces not only the overall positive charge of the protein but also changes its hydrophobic/hydrophilic properties. It, therefore, would be reasonable to suspect that

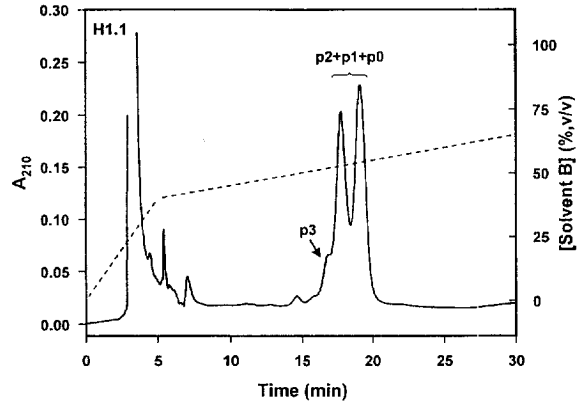

Fig. 3. Cation-exchange chromatography of multi-phosphorylated histone H1.1. The histone H1.1 fraction isolated by RP-HPLC (Fig. 1) was analysed on a PolyCAT A column (see Section 2.7). Abbreviations used were the same as for Fig. 2.

phosphorylated histones could also be separated by RP-HPLC. However, as shown in Fig. 1, phosphorylated and unmodified H1 histones coeluted in RP-HPLC. An explanation for this somewhat unexpected result might be the special structure of the H1 histone molecule: an extended hydrophobic domain is flanked by two hydrophilic basic regions. We assume that the interaction dominantly occurs between the hydrophobic column matrix and the central hydrophobic domain of the protein. Since phosphorylation takes place exclusively in the hydrophilic N- and C-terminal regions of the histones the binding behaviour is affected to a lesser extent. In contrast, normal-phase chromatography (e.g., HILIC) is based mainly on polar interactions and for this reason should be more suitable for separating phosphorylated H1 histones. Therefore, in order to resolve these modified histone proteins we employed the same PolyCAT A column primarily used for IEC, but chromatographed in the presence of acetonitrile. Acetonitrile promotes hydrophilic interactions with the hydrophilic stationary phase, these hydrophilic interactions becoming increasingly dominant in the separation procedure as the acetonitrile concentration is increased. It should be noted that the poor solubility of phosphate in mobile phases containing high concentrations of acetonitrile requires the use of a MPA–TEA (pH 3.0) buffer system, thus enabling the use of higher salt concentrations and, consequently, diminishing the elution time of the histone sample. Fig. 4 shows the separation of histone H1.1 under IEC conditions with 0% (Fig. 4a), 40% (Fig. 4b) and 70% (Fig. 4c) acetonitrile. The addition of 70% acetonitrile to the mobile phase, typical HILIC conditions, yields optimal resolution: four major and a number of minor peaks were observed. In addition, the histone separation was accomplished in less than 40 min. The first peak eluting at about 10 min was identified as protamine sulphate. This reagent was generally added to the histone H1 fractions isolated by RP-HPLC in order to stabilize the proteins. The (minor) second peak which eluted at approximately 15 min represents an impurity and not an H1 histone. Based on the peak pattern obtained in Fig. 2 we supposed that the four peaks eluting between 18 and 40 min correspond to the non-, mono-, di- and tri-phosphorylated H1.1. In order to verify this assumption, the histone H1.1

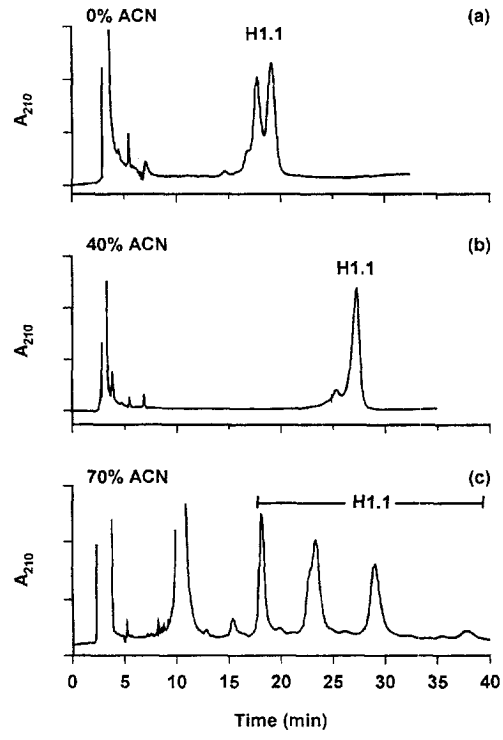

Fig. 4. Influence of acetonitrile on the resolution of multi-phosphorylated histone H1.1. The histone H1.1 fraction isolated by RP-HPLC (Fig. 1) was analysed on a PolyCAT A column (see Section 2.7) (a) in the absence of acetonitrile (b) in the presence of 40% acetonitrile and (c) in the presence of 70% acetonitrile.

subfraction used for the HILIC run shown in Fig. 3 was first digested with alkaline phosphatase. After treatment using the enzyme, the sample was subjected to HILIC. The result is illustrated in Fig. 5. Comparison with Fig. 4c shows a total loss of the two later eluted peaks and a dramatic decrease in the peak eluted at about 24 min. We therefore assume that the peak eluting at about 19 min corresponds to the non-phosphorylated H1.1 (designated p_0), and the second peak eluting at 24 min (p_1) is monophosphorylated H1.1, not completely digested by the enzyme. In this context it should be noted that in Fig. 4c the peak for H1.1 (p_1) has a shoulder on the front. This shoulder which we have found repeatedly is most likely due to phosphorylation at two alternative locations. Moreover, investigations have revealed (data not shown) that alkaline phosphatase has easier access to one of the two alternative phosphorylation sites. This is evident in Fig. 5, where only a single

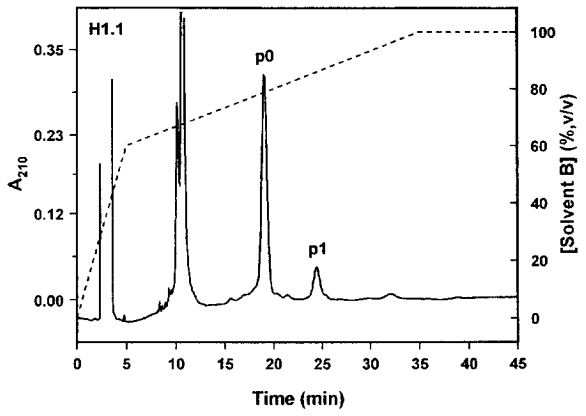

Fig. 5. Removal of phosphate from histone H1.1 by alkaline phosphatase. Multi-phosphorylated histone H1.1 was isolated by RP-HPLC (Fig. 1), digested with alkaline phosphatase (see Section 2.6 Section 2.9) and subjected to HILIC. HILIC conditions same as in Fig. 4c; p_0, p_1=non- and mono-phosphorylated histone H1.1.

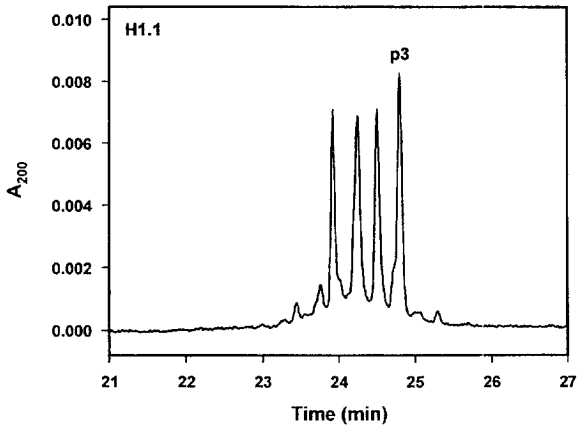

Fig. 6. Identification of phosphorylated histone H1.1 subfractions by HPCE. The histone H1.1 fraction eluted at 38 min in the HILIC run (Fig. 4c) was added to the histone H1.1 fraction isolated by RP-HPLC (Fig. 1) and subjected to CE. CE conditions were the same as for Fig. 2; p_3=tri-phosphorylated histone H1.1.

product is present in trace amounts for the p_1 peak. At present, the effects of site-specific phosphorylation on the HILIC separation are under investigation in our laboratory.

Confirmatory assignment of the four fractions obtained by HILIC (Fig. 4c) was performed by CE by adding individual HILIC fractions to the whole histone H1.1 sample leading to a characteristic increase of one peak in the electropherogram (shown in Fig. 6 for the unknown peak eluted at 38 min in Fig. 4c).

All these procedures permitted the assignment of histone H1.1 peaks shown in Fig. 7. The more positively-charged non-phosphorylated histone H1.1 ($H1.1p_0$) was eluted first, followed by the less positively-charged mono- ($H1.1p_1$), di- ($H1.1p_2$) and tri- ($H1.1p_3$) phosphorylated forms of histone H1.1. Thus, the peak order is the same as in CE (Figs. 2 and 6), but the inverse of that in cation-exchange chromatography (Fig. 3), where proteins are eluted in order of increasing net positive charge. This indicates that a hydrophilic interaction mechanism is the dominant separation process. In the HILIC mode proteins are eluted in order of increasing hydrophilicity, and phosphorylation of proteins increases their hydrophilicity. The partial transition from an IEC to a HILIC mechanism is clearly demonstrated in Fig. 4b. With 40% acetonitrile, hydrophilic interactions are superimposed on electrostatic interactions to an extent that the inversion in elution order is incomplete; all these proteins have nearly the same retention time. Also worth noting is that in the HILIC mode, in contrast to IEC, we did not observe any differential resolution of phosphorylated H1 histones depending on the PolyCAT A column utilized. Finally, it should be noted that this method is not limited to the separation of histone H1 proteins; on the contrary, we have successfully

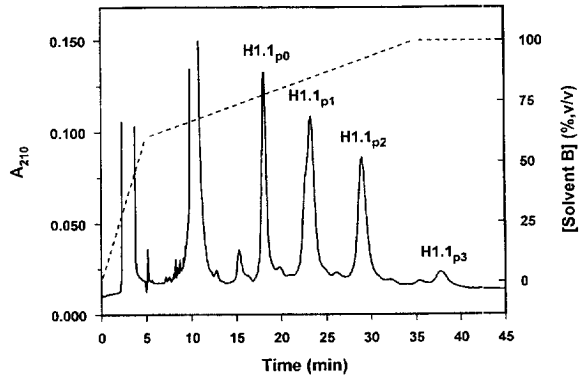

Fig. 7. Separation of multi-phosphorylated histone H1.1 of Raji cells by HILIC. The histone H1.1 fraction used was isolated by RP-HPLC (Fig. 1) and subjected to HILIC. HILIC conditions as in Fig. 4c; $H1.1p_0$, $H1.1p_1$, $H1.1p_2$ and $H1.1p_3$=non-, mono-, di- and tri- phosphorylated histone H1.1.

applied the HILIC method presented for the fractionation and isolation of a number of other phosphorylated proteins and peptides (data not shown).

The combined RP-HPLC–HILIC system described here represents for the first time a fast, reliable and efficient chromatographic separation method for the fractionation of multi-phosphorylated histone H1 subtypes into their non-phosphorylated and distinct phosphorylated forms. The method allows both semi-preparative and analytical application. Compared to traditional IEC, the HILIC technique has shorter separation times and, in addition, superior resolution. Its application will be exceedingly useful in studying biological function(s) of histone phosphorylation in future.

Acknowledgments

This work was supported by the Oesterreichische Nationalbank, Jubilaeumsfondprojekt 6042. We are very grateful to R. Berberich and A. Devich for their excellent technical assistance.

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