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Separation of phosphorylated histone H1 variants by high-performance capillary electrophoresis

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ABSTRACT

High-performance capillary electrophoresis (HPCE) was used to separate successfully distinct phosphorylated derivatives of individual histone H1 variants. With an untreated capillary (50 cm × 75 μm I.D.) the electrophoresis was performed in about 15 min. Inconvenient interactions of these highly basic proteins with the capillary wall were eliminated by using 0.1 *M* sodium phosphate buffer (pH 2.0) containing 0.03% hydroxypropylmethylcellulose. Under these experimental conditions the histone H1 variants H1b and H1c obtained from mitotic enriched NIH 3T3 fibroblasts and isolated by reversed-phase high-performance liquid chromatography were clearly separated in their non-phosphorylated and different phosphorylated forms. This result was confirmed by acid–urea gel electrophoresis, comparison with non-phosphorylated histones H1b and H1c, isolated from quiescent NIH 3T3 cells, and incubation of multi-phosphorylated histone H1b with alkaline phosphatase and subsequent acid–urea and capillary electrophoresis. The results illustrate that the application of HPCE to the analysis of histone modifications provides a new alternative to traditional gel electrophoresis.

INTRODUCTION

One of the most widely utilized electrophoretic methods for the study of histones and histone modifications has been polyacrylamide gel electrophoresis [1–4]. All methods of gel electrophoresis, however, have shortcomings, *e.g.*, the preparation and the staining and destaining of gels is labour intensive and time consuming. In addition, with low sample concentrations, specialized staining procedures are often required. Finally, gel electrophoretic methods are unsatisfactory for precise quantification.

High-performance capillary eletrophoresis (HPCE), combining the substantial advantages of conventional gel electrophoresis and high-performance liquid chromatography, was introduced by Jorgenson and Lukacs [5] and has opened up new prospects for the separation of biomolecules. In HPCE, compounds are resolved according to their ability to migrate in an electric field inside a fused-silica capillary. Nanolitre volumes or less of sample can be separated and, in contrast to common gel electrophoresis, rapid, quantitative, fully automat-

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ed and highly efficient analyses can be achieved. Although HPCE is still in its infancy, we have previously shown the application of this technique to the separation of core histones and their acetylated modifications [6].

In this paper, we describe an efficient and rapid method for analysing phosphorylated histone H1 variants by HPCE using untreated fused-silica capillaries.

EXPERIMENTAL

Chemicals

Hydroxypropylmethylcellulose (HPMC) (4000 cP) and trifluoroacetic acid (TFA) were obtained from Sigma (Munich, Germany), ethylene glycol monomethyl ether (EGME) from Aldrich (Steinheim, Germany), Triton X-100, Tris and phenylmethanesulphonyl fluoride (PMSF) from Serva (Heidelberg, Germany) and colcemid, foetal calf serum (FCS), Dulbecco's minimum essential medium (DMEM), phosphate-buffered saline (PBS) and HAM's medium F12 from Bochringer (Mannheim, Germany). All other chemicals were purchased from Merck (Darmstadt, Germany).

Cell line and culture conditions

Mouse NIH 3T3 fibroblasts were grown in monolayer cultures and cultivated in DMEM supplemented with 10% FCS, penicillin (60 μg/ml) and streptomycin (100 μg/ml) in the presence of 5% CO_2. For synchronization, cells were seeded at a density of $4 \cdot 10^5$ cells per dish (48 cm^2) and grown for 12 h in normal supplemented DMEM. The cells were then washed once with prewarmed PBS and then incubated in starvation medium (a 1:1 mixture of HAMs medium F12 and DMEM) supplemented with 0.1% FCS for 72 h to accumulate the cells in G0/G1 phase. To release the cells from the G0/G1 phase arrest, fresh medium supplemented with 10% FCS was added. After 18 h of stimulation, colcemid (0.06 μg/ml) was added to the cultures for 6 h, then the cells were harvested.

Isolation of whole histones

G0/G1 phase- and colcemid-treated cells were washed twice with ice-cold PBS and incubated in ice-cold lysis buffer [0.05 *M* Tris–HCl (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* phenylmethanesulphonyl fluoride–0.1% Triton X-100] for 5 min and were removed from the dish with a "rubber policeman". Nuclei were pelleted at 2500 *g* for 15 min at 4°C and washed with lysis buffer without Triton X-100. Whole histones were isolated from the resulting nuclear preparation by extraction with 0.2 *M* H_2SO_4 at 4°C for 1 h. After centrifugation at 10 000 *g* in a microfuge for 20 min, the supernatant was mixed with five volumes of chilled acetone–HCl (98:2). After 12 h, the precipitated histones were centrifuged at 10 000 *g* for 20 min, washed twice with pure acetone, dissolved in water containing 0.01 *M* 2-mercaptoethanol and lyophilized.

High-performance liquid chromatography

The equipment used consisted of two Model 114M pumps, a Model 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. The protein separations were performed on a column (125 mm × 8 mm I.D.) filled with Nucleosil 300-5 C_4 (Machery–Nagel, Düren, Germany). The lyophilized proteins were dissolved in water containing 0.1% of trifluoroacetic acid and samples of 100 μg of histones were injected on to the column. At a constant flow-rate of 1 ml/min the H1 histones were eluted within 35 min using a linear gradient from 41 to 61% B (solvent A = water containing 10% of EGME and 0.1% of TFA, solvent B = 10% EGME–70% acetonitrile–0.1% TFA).

Polyacrylamide gel electrophoresis

Histone fractions from HPLC runs were collected, lyophilized and stored at −20°C. Histones H1 were analysed by SDS polyacrylamide gel electrophoresis (PAGE) (15% polyacrylamide, 0.1% SDS) as described by Laemmli [1], and by acid-urea (AU) PAGE (15% polyacrylamide, 0.9 *M* acetic acid, 2.5 *M* urea) according to Lennox *et al.* [4]. The gels were stained with 0.1% Serva Blue R in 40% ethanol–5% acetic acid and destained overnight in 20% ethanol–5% acetic acid.

Incubation of histones H1 with alkaline phosphatase

To prevent the appearance of phosphorylated

bands in AU-PAGE and in HPCE, the isolated histones were incubated with alkaline phosphatase. About 100 μg of whole histones in 0.25 ml of 0.01 *M* Tris–HCl (pH 8.0) and 0.001 *M* phenylmethylsulphonyl fluoride were mixed with 210 μg of *Escherichia coli* alkaline phosphatase (60 units/mg; Sigma) for 12 h at 37°C according to Sherod *et al.* [7].

Capillary electrophoresis

HPCE was performed on a Beckman system P/ACE 2100 controlled by an AT386 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 fitted with 75 μm I.D. fused silica of 58.8 cm total length (50 cm to the detector). Protein samples (concentration 0.5 mg/ml) were injected by pressure for 5 s and on-column detection was performed by measuring UV absorption at 200 nm. An untreated capillary was used in all experiments, but after every 5–10 injections the capillary was rinsed with water, 0.1 *M* NaOH, water, 0.5 *M* H_2SO_4, water and finally with the running buffer. Washing with each solvent was applied for 2 min. Runs were carried out in 0.1 *M* phosphate buffer (pH 2.0) containing 0.03% HPMC at constant voltage (16 kV) and a capillary temperature of 20°C.

RESULTS AND DISCUSSION

The utility of HPCE for the separation of small molecules has been demonstrated [8–10]. However, the application of this new technique to the analysis of proteins can be problematic as they often tend to interact with the negatively charged silanol groups of the capillary wall. These interactions result in band broadening and tailing with significantly reduced separation efficiency and a non-linear relationship between measured absorption values and analyte concentration. Such non-specific adsorption effects may be overcome by the use of buffer pH values higher than the p*I* of proteins [11], the use of low-pH buffer systems [12], the application of dynamic coating agents, which interact weakly with the capillary surface and are generally added to the separation buffer [13], and chemical derivatization of the silanol groups [5,14,15]. Combinations of these systems have also been described [16].

For the analysis of core histones and their acetylated modifications, we have recently described an efficient HPCE method [6]. In this work, we have investigated the potential utility of HPCE for the separation of distinct phosphorylated linker histone variants. For this purpose, whole histones were extracted from mitotic enriched mouse NIH 3T3 fibroblasts containing highly phosphorylated H1 his-

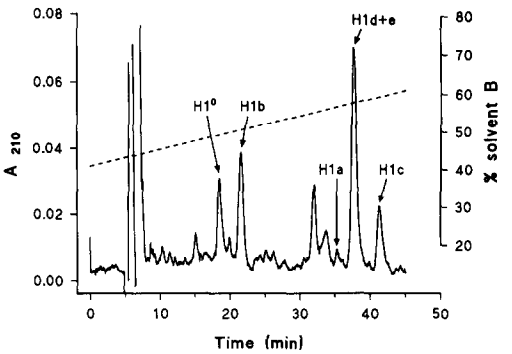

Fig. 1. Separation of H1 histones by RP-HPLC. Amounts of 100 μg of protein samples isolated from mitotic enriched NIH 3T3 fibroblasts were injected on to a Nucleosil 300-5 C_4 column. Flow-rate, 1 ml/min. A linear acetonitrile gradient was used with an increase in solvent B (70% acetonitrile) from 43 to 63% within 45 min. Absorbance was monitored at 210 nm. The purified H1 subtypes were lyophilized and analysed by HPCE and PAGE.

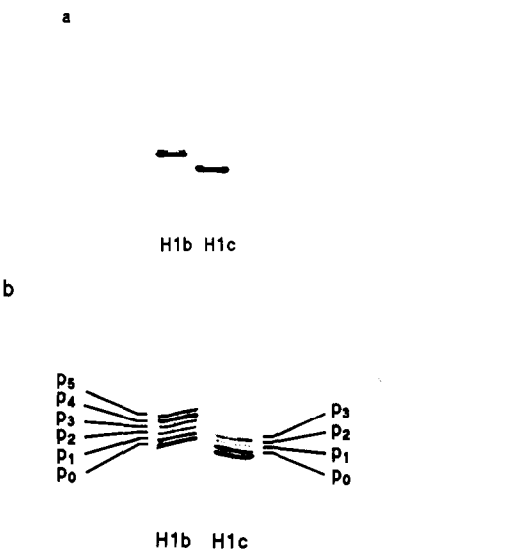

Fig. 2. Gel electrophoresis of multi-phosphorylated histone H1 variants separated by the HPLC system used in Fig. 1. (a) SDS gel electropherogram; (b) AU gel electropherogram.

tone variants. In order to obtain these H1 subtypes we used reversed-phase (RP) HPLC, which has been demonstrated to be an excellent technique for the separation of the very lysine-rich histones [17–19]. As shown in Fig. 1, five H1 subfractions were clearly separated from each other: histone $H1^0$, H1b, H1a, a mixture of H1d and H1e and finally H1c. For our investigations we used the subtypes H1b and H1c, which are known to be phosphorylated in interphase and mitotic cells to a larger extent [20]. The purity of the histone samples was checked by SDS-PAGE (Fig. 2a). Neither fraction H1b nor H1c is contaminated with any other proteins, but they consist of a mixture of non-phosphorylated and different phosphorylated forms which are resolved in acid–urea gels (Fig. 2b). These modified protein variants, however, cannot be separated by RP-HPLC. It should be mentioned that the minor band migrating just above the main component in Fig. 2a represents phosphorylated histone H1b, whose shift is caused by a conformational change of the modified histone [4]. The histone fractions H1b and H1c obtained by HPLC (Fig. 1) were subjected to capillary electrophoresis.

Fig. 3a and b show the HPCE runs of the histone fractions H1b and H1c, respectively, obtained by HPLC (Fig. 1). The parent histone variants and the different phosphorylated derivatives were clearly separated in 0.1 *M* phosphate buffer (pH 2.0) containing 0.03% HPMC within about 15 min. The histone fraction H1b (Fig. 3a) was baseline separated into six peaks: H1b-p_0, the fastest migrating component representing the non-phosphorylated H1b variant, followed by five different phosphorylated forms designated H1b-p_1 to H1b-p_5. The number of peaks in the electropherogram (Fig. 3a) corresponds to the number of bands in the acid–urea gel (Fig. 2b, left) In addition, the relative amounts

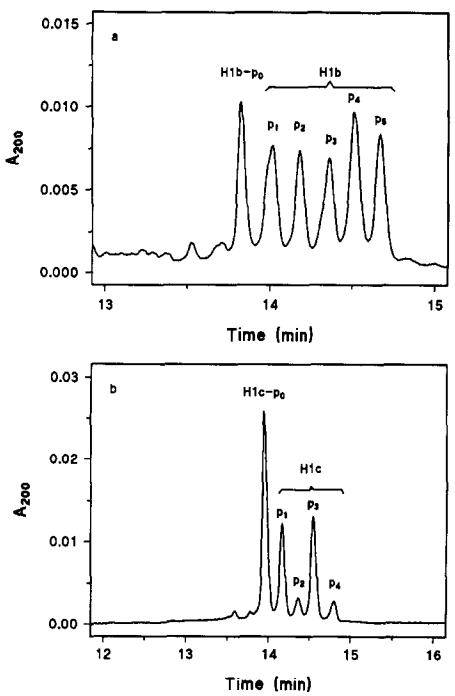

Fig. 3. HPCE of multi-phosphorylated histone H1 variants in a 50 cm × 75 μm I.D. untreated capillary. Samples were injected for 5 s by pressure. Electrophoresis was performed at 16 kV in 0.1 *M* phosphate buffer (pH 2.0) containing 0.03% HPMC. (a) HPCE of non-phosphorylated histone H1b (H1b-p_0) and distinct phosphorylated forms of H1b (H1b-p_1 to H1b-p_5); (b) HPCE of non-phosphorylated histone H1c (H1c-p_0) and distinct phosphorylated forms of H1c (H1c-p_1 to H1c-p_4).

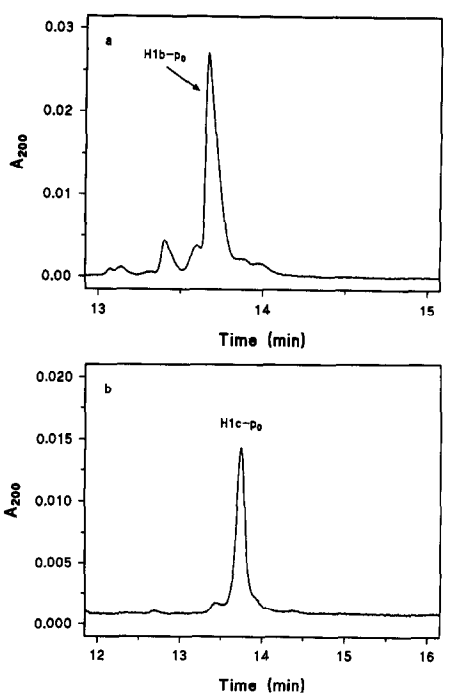

Fig. 4. HPCE of non-phosphorylated histone H1 variants in a 50 cm × 75 μm I.D. untreated capillary. Electrophoretic conditions as in Fig. 3. (a) HPCE of non-phosphorylated histone H1b (H1b-p_0); (b) HPCE of non-phosphorylated histone H1c (H1c-p_0).

H1b H1c

Fig. 5. AU gel electrophoresis of non-phosphorylated histone H1 variants. Non-phosphorylated histone variants H1b and H1c were isolated from quiescent NIH 3T3 fibroblasts by RP-HPLC as in Fig. 1 and subjected to AU gel electrophoresis.

of the individual peaks coincide with those of the bands. The histone fraction H1c consisting of four bands in the AU gel (Fig. 2b, right) was separated by HPCE (Fig. 3b) into five peaks, a non-phosphorylated (H1c-p_0) and four phosphorylated H1 derivatives (H1c-p_1 to H1c-p_4).

In order to prove the existence of multi-phosphorylated forms of histones H1b (Fig. 3a) and H1c (Fig. 3b), we analysed these two histone H1 variants obtained from quiescent NIH 3T3 fibroblasts by HPLC (data not shown) and subsequently by HPCE. As slowly or non-dividing cells contain H1 histones primarily in their non-phosphorylated form [7], we expected only a single peak in the corresponding electropherogram. In fact, we found that both histone fractions H1b and H1c consist of a single peak each, designated H1b-p_0 (Fig. 4a) and H1c-p_0 (Fig. 4b), respectively. The purity of these

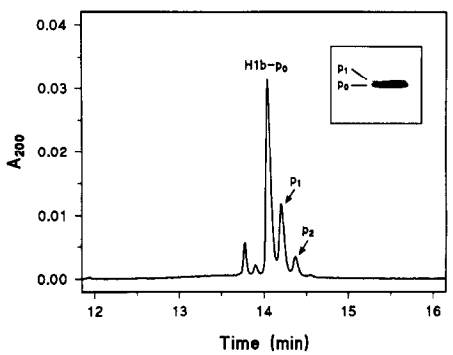

Fig. 6. Removal of phosphate from histone H1b by alkaline phosphatase. Multi-phosphorylated histone variant H1b was isolated from mitotic enriched NIH 3T3 fibroblasts RP-HPLC as in Fig. 1, digested with alkaline phosphatase as described by Sherod *et al.* [7] and subjected to HPCE. Electrophoretic conditions as in Fig. 3. The inset shows the corresponding analysis by AU gel electrophoresis. H1b-p_0 = Non-phosphorylated histone H1b; H1b-p_1 and H1b-p_2 = distinct phosphorylated forms of histone H1b.

histone variants was checked by AU-PAGE (Fig. 5).

In a further experiment, the same material as used for the HPCE analysis shown in Fig. 3a was digested with alkaline phosphatase. After treatment with the enzyme, the sample was chromatographed (data not shown) and subjected to HPCE (Fig. 6). In contrast to Fig. 3a, only three peaks of histone H1b were detectable: one prominent peak consisting of non-phosphorylated histone H1b (designated H1b-p_0) and two minor peaks of different phosphorylated histones H1b (p_1 and p_2). Therefore, the total loss of the highly phosphorylated forms of H1b-p_3 to H1b-p_5 and the dramatic decrease in H1b-p_2 and H1b-p_1 confirm our assignment in Fig. 3.

CONCLUSION

The first application of HPCE to the analysis of phosphorylated H1 histones has been described. With an untreated capillary and a phosphate buffer system (pH 2.0) containing the dynamic coating agent hydroxymethylpropylcellulose, remarkable results were obtained. This new technique permits an excellent resolution of non-phosphorylated and different phosphorylated H1 histone variants within about 15 min and therefore provides an important alternative to the traditional gel electrophoresis.

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