Possible role of histone acetylation and histone H1^o replacement for the initiation of replication in regenerating rat liver

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The role of histone acetylation and DNA synthesis has been investigated extensively in the regenerating rat liver system in the presence and absence of the cyclophosphamide derivative mafosfamide. We demonstrate a mafosfamide-induced inhibition of maximum histone acetyltransferase activity followed by a second elevation of enzyme activity and an accompanying total suppression of DNA synthesis for 7–8 h. The maximum of histone acetyltransferase activity, in parallel with an elevated acetylation *in vivo*, the consecutive replacement of histone H1^o and initiation of replication occur sequentially in the presence and absence of mafosfamide, but with a temporary delay of 7–8 h. Our data indicate that modifications of histone acetyltransferase (EC 2.3.1.48) activity do not significantly influence the acetylation patterns of histones H3 and H4. The mafosfamide-induced change of histone acetyltransferase activity and acetylation *in vivo*, the shift of histone H1^o exchange and the consecutive transition of initiation of replication suggest that these three events might be functionally related.

INTRODUCTION

The nucleosome is the fundamental basic subunit of eukaryotic chromatin [1,2], and reversible modifications of histones have been suggested to alter nucleosomal structure within the cell cycle. One of these heterogeneity-producing events, catalysed by the enzyme histone acetyltransferase (EC 2.3.1.48), is the reversible post-translational acetylation of lysine residues at the basic N-terminal domains of the core histones (for reviews, see [3,4]). It has already been shown that histone acetylation alters the solubility and stability of the nucleosome and its higher-order structures by influencing the mainly electrostatic histone–DNA interactions [5–7]. However, the function of this reversible dynamic process is poorly understood, and histone acetylation has been correlated with transcription [8,9], replication [10,11], differentiation [12] or DNA repair [13]. Several reports suggested that certain patterns of acetylation might be typical for specific alterations of chromatin due to various cellular processes [14–16].

Previous data obtained by our group demonstrated that maximum histone acetylation, H1^o replacement and initiation of replication occur sequentially in the regenerating rat liver model [17]. On the basis of these results we decided to investigate whether enhanced histone acetylation and impairment of histone H1^o are necessary for the initiation of replication. For this aim we used mafosfamide, a derivative of cyclophosphamide activated directly in the cell without previous biotransformation in the liver [18], which is known to depress histone acetylation and DNA synthesis [19–22]. We investigated its effects on the activity of histone acetyltransferase, acetylation *in vivo*, DNA synthesis, the quantitative behaviour of histone H1^o and the acetylation patterns of histones H3 and H4 in the regenerating rat liver system.

MATERIALS AND METHODS

Preparation of nuclei

Male Sprague–Dawley rats (between 250 and 330 g body wt. each) were partially hepatectomized or sham-operated under diethyl ether anaesthesia as previously described [23]. The alkylating agent mafosfamide (ASTA Z7557; 4-sulphonatoethylthiocyclophosphamide) was injected intraperitoneally at a concentration of 2×10^{-4} mol/kg at the time of partial hepatectomy. Mafosfamide, which was a gift from ASTA-Werke AG, Bielefeld,

Germany, was dissolved in water immediately before injection. The livers were isolated at various times after operation and placed in 0.14 M-NaCl, homogenized in 3 vol. of STKM (0.25 M-sucrose/25 mM-KCl/5 mM-MgCl₂/50 mM-Tris/HCl, pH 7.9) with a Potter homogenizer. The cells were sedimented by centrifugation at 1000 *g* and washed again using STKM in the same way. The cells were suspended in STKM containing 0.1 % Nonidet P40, Dounce-homogenized several times and nuclei were pelleted at 1000 *g*. Nuclei were washed twice with STC (0.34 M-sucrose/2 mM-CaCl₂/100 mM-Tris/HCl, pH 7.4) and suspended in the same buffer at a concentration of 20 mg of protein/ml. All preparations of liver cells were done on ice. All buffers contained 0.1 % 2-mercaptoethanol (v/v), 0.1 mM-phenylmethanesulphonyl fluoride and 10 mM-sodium butyrate to inhibit histone deacetylase [24].

Acetylation of histones in isolated nuclei

Nuclei [$(1.2\text{--}1.5) \times 10^7$; approx. 1.2 mg of protein] were pre-incubated in STC with 100 μ M unlabelled acetyl-CoA at 22 °C for 5 min (total volume 200 μ l). The reaction was started by addition of [³H]acetyl-CoA (1.6 Ci/mmol; final concn. 3 μ M) and terminated after 0, 1, 2, 4 and 8 min at 22 °C by addition of 1 ml of ice-cold STC containing 200 μ M unlabelled acetyl-CoA and subsequent centrifugation. Histones were extracted with 0.2 M-H₂SO₄, precipitated with 40 % (v/v) trichloroacetic acid and dissolved in 0.1 M-NaOH. Radioactivity was measured by using a liquid-scintillation counter, and histone protein concentration was determined by the Lowry method [25]. Acetyltransferase specific activities is expressed as pmol/min per mg of histone. Histones were analysed by 8 M-urea/0.46 % Triton X-100/PAGE, stained with Coomassie Blue, and the fluorographs of the gels were performed as described by Bonner *et al.* [26]. The relative amounts of acetylation of histone subtypes according to the blackening of the film were calculated by using a spectrophotometer at 700 nm.

Measurement of acetylation *in vivo* of histones

Rats were injected with 1 mCi of [³H]acetic acid (4.34 Ci/mmol) per 100 g of rat 30 min before isolation of the livers. Nuclei were prepared and histones were isolated and precipitated as described above. Radioactivity was determined by a liquid-scintillation counter and, after protein determination by Lowry's method [25], the acetylation *in vivo* of histones was

Fig. 1. Uptake of [^3H]acetate into core histones

Rat liver nuclei, labelled with [^3H]acetate *in vivo* for 30 min, were isolated; histones were extracted and analysed by 8 M-urea/0.46 % Triton X-100/PAGE. After staining with Coomassie Blue, a fluorographic determination was carried out as previously described [26]. The Figure shows the result of a representative experiment. (a) Coomassie Blue-stained gel; (b) fluorography of the same gel.

calculated as c.p.m./mg of histone. To investigate whether the alterations of [^3H]acetate incorporation are due to acetylation of core histones and do not reflect changes in protein and histone synthesis, the acetate-labelled histones were further analysed by 8 M-urea/0.46 % Triton X-100/PAGE, and fluorography was performed as described by Bonner & Laskey [26]. Fig. 1 shows a representative Coomassie Blue-stained gel and the fluorograph of the same gel of acetate-labelled histones. It was demonstrated that [^3H]acetate is only incorporated into core histones and not taken up by other proteins or histone H1, indicating that the incorporation of [^3H]acetate *in vivo* specifically reflects post-translational histone acetylation.

Determination of DNA synthesis

Hepatectomized rats were labelled by intraperitoneal injection of 1 μCi of [^3H]thymidine (22 Ci/mmol)/g of rat. Livers were harvested after 30 min, and nuclei were prepared as described above. Nuclei were resuspended in STC and washed with 5 % (v/v) trichloroacetic acid and 100 % ethanol. The pellet was dissolved in 0.25 M-KOH and the DNA concentration was determined as published by Keck [27]. Radioactivity was measured as described above, and the specific activity of DNA was calculated.

Preparation of H1 histones

H1 histones were extracted from rat liver nuclei with 5 % (v/v) perchloric acid for 1 h on ice and precipitated by using 3.5 vol. of acidified acetone at -20°C overnight. After centrifugation at 20000 g for 30 min, the sediments were washed twice with ice-cold acetone. Histones were freeze-dried and kept at -20°C . H1 histones were dissolved in SDS sample buffer and separated by SDS/PAGE as described by Weintraub *et al.* [28]. Gels were stained with Coomassie Blue, and the relative amounts of H1 histones on each lane were determined by using a spectrophotometer at 572.5 nm.

RESULTS

Influence of mafosfamide on DNA synthesis and histone acetylation

Initiation of replication occurs in the regenerating rat liver system, as pointed out in Fig. 2, between 16 and 18 h after partial

hepatectomy. Under the influence of mafosfamide, given via intraperitoneal injection at the time of operation, we observed a total inhibition of [^3H]thymidine incorporation, together with a loss of increase of DNA amounts at these time points. This complete retardation of replication lasted for approx. 7 h, and was followed by the initiation of DNA synthesis, which reached a first peak 26 h after partial hepatectomy, comparable with the maximum of DNA synthesis observed in the absence of mafosfamide (Fig. 2).

Secondly, we examined the influence of mafosfamide on the first maximum of histone acetyltransferase activity, which occurs 13 h after partial hepatectomy. Application of mafosfamide, as described above, resulted in a decrease in enzyme activity from 71 ± 6 to 41 ± 5 % (means $\pm$ s.d. for three experiments performed in duplicate) compared with sham-operated liver (0 %) used as control (Fig. 2). Afterwards, histone acetylation in mafosfamide-treated livers decreased to lower values, but rose again after a short period and reached a second and higher maximum of enzyme activity (62 ± 4 %) 21 h after partial hepatectomy, just before the onset of DNA synthesis. At these time points, from 17 to 21 h after operation, enzyme activities within mafosfamide-treated livers surpass the amounts of histone acetylation estimated in regenerating liver in the absence of this alkylating agent (Fig. 2).

In all these cases the kinetics of histone acetyltransferase were monophasic and, additionally, the application of mafosfamide

Fig. 2. Histone acetylation and DNA synthesis in regenerating rat liver in the presence or absence of mafosfamide

Nuclei were isolated from regenerating rat liver several hours after partial hepatectomy and incubated with [^3H]acetyl-CoA at 22°C for 0, 1, 2, 4 or 8 min. The reaction was stopped by addition of unlabelled acetyl-CoA, histones were isolated and specific radioactivity was measured with a liquid-scintillation counter. Activities of histone acetyltransferase were estimated as pmol/min per mg of histone. Relative percentage deviations of enzyme activities in rat liver regenerated in the presence (■) or absence (□) of mafosfamide, injected at the time of partial hepatectomy, from the sham-operated control are shown. The control corresponds to 0.219 ± 0.026 pmol/min per mg of histone. After various times of regeneration, rat livers were labelled with [^3H]thymidine *in vivo* for 30 min, and nuclei were isolated and washed with trichloroacetic acid and ethanol. Radioactivity was measured in a liquid-scintillation counter, and DNA concentrations were determined as described by Keck [27]. DNA synthesis in regenerating rat liver in the presence (●) or absence (○) of mafosfamide is relative to the sham-operated control, for which the value is 8450 ± 720 c.p.m./mg of DNA. Each point represents the mean value from three separate experiments performed in duplicate. Control data are expressed as means $\pm$ s.d. for 15 experiments.

Fig. 3. Acetylation of histones *in vivo* in regenerating rat liver nuclei influenced by mafosfamide

Rat liver nuclei were isolated and purified 30 min after labelling *in vivo* with [3 H]acetic acid. Histones were extracted, radioactivity was measured and specific acetylation was estimated as c.p.m./mg of histone. The various states of acetylation were compared in the absence (○) or presence (●) of mafosfamide, injected at the time of operation. Acetylation *in vivo* is expressed as percentage deviation from that of the sham-operated control (= 0%) which corresponds to 5428 ± 596 c.p.m./mg of histone. Each point represents the mean $\pm$ S.D. for three experiments performed in duplicate.

Table 1. Relative percentage of incorporation *in vitro* of [3 H]acetyl-CoA into histones H3 and H4, isolated from regenerating rat livers partially influenced by mafosfamide, at time points of maximum acetylation

Nuclei were isolated from regenerating and sham-operated (= control) rat livers 13 and 18 h after partial hepatectomy or sham operation. Nuclei were labelled with [3 H]acetyl-CoA *in vitro*, histones were extracted, analysed by 8 M-urea/0.46% Triton/PAGE and fluorography was performed as described in [26]. The fluorographs were evaluated with a spectrophotometer (at 700 nm), and the relative amounts of [3 H]acetyl-CoA uptake of the several histone H3 and H4 subtypes (H4.1–H4.4, i.e. monoacetylated H4 to tetraacetylated H4) were calculated according to the blackening of the film. Data are shown as relative percentage amounts of total histone H3 and H4 acetylation, i.e. H3 + H4 = 100%, and are expressed as means $\pm$ S.D. for three experiments and six in the case of controls. The term 'Reg.-time' reflects the time in h after partial hepatectomy in presence (+) or absence (–) of mafosfamide ($2 \cdot 10^{-4}$ mol/kg) injected at time points of operation.

Reg.-time (h)	Acetylation (% of total H3 and H4 acetylation)				
	H3 (total)	H4.1	H4.2	H4.3	H4.4
13 (+)	53.5 $\pm$ 1.3	15.4 $\pm$ 0.7	10.6 $\pm$ 0.4	8.2 $\pm$ 0.9	9.0 $\pm$ 0.8
13 (–)	58.7 $\pm$ 2.1	12.7 $\pm$ 1.9	11.0 $\pm$ 0.2	7.7 $\pm$ 0.7	6.2 $\pm$ 0.1
18 (+)	55.0 $\pm$ 1.4	12.6 $\pm$ 2.5	9.9 $\pm$ 1.0	8.0 $\pm$ 0.3	10.6 $\pm$ 0.3
18 (–)	51.6 $\pm$ 1.7	16.6 $\pm$ 1.1	11.6 $\pm$ 0.5	8.1 $\pm$ 0.4	8.2 $\pm$ 0.6
Control (+)	53.1 $\pm$ 1.4	14.4 $\pm$ 0.7	12.1 $\pm$ 0.8	8.0 $\pm$ 1.1	9.3 $\pm$ 1.1
Control (–)	53.4 $\pm$ 1.7	16.1 $\pm$ 1.3	12.8 $\pm$ 1.1	8.5 $\pm$ 0.9	7.2 $\pm$ 0.7

had no significant effect on enzyme activities in sham-operated livers.

Thirdly, we administered [3 H]acetate *in vivo* in order to investigate the acetylation pattern *in vivo* in the presence or absence of mafosfamide. We found that the results of the measurement of histone acetyltransferase activity *in vitro* are in good accordance with histone acetylation *in vivo* (Fig. 3).

Fig. 4. Alterations of amounts of histone H1° during regeneration in the presence and absence of mafosfamide

Total histone H1 was extracted with 5% (v/v) perchloric acid from purified nuclei isolated from regenerating rat liver several hours after partial hepatectomy. Mafosfamide (2×10^{-4} mol/kg) was injected at the time of operation. Histone H1 subtypes were analysed by SDS/PAGE, stained with Coomassie Blue and the relative amounts were estimated spectrophotometrically at 572.5 nm. Relative amounts of histone H1° (i.e. H1° as a percentage of the total amount of histone H1) are plotted against DNA synthesis *in vivo* (see Fig. 2), estimated in both the presence (■, ●) and absence (□, ○) of mafosfamide. Each point represents the mean value for three experiments performed in duplicate. The reduction of amounts of histone H1° after 16 h in the absence (–mf) and after 23 h in the presence of mafosfamide (+mf) were statistically significant. The calculation was done by Student's *t* test: H1° (+mf), $P < 0.025$; H1° (–mf), $P < 0.001$.

Increases or decreases of the activity of histone acetyltransferase *in vitro* are paralleled by an enhancement of, or reduction in, histone acetylation *in vivo* (cf. Figs. 2 and 3).

Acetylation patterns of histones H3 and H4 during enzyme activity maxima

We investigated the patterns of acetylation of the histones H3 and H4 at the time of maximum acetyltransferase activities by using Triton/PAGE, fluorography and spectrophotometry. Table 1 demonstrates that the relative acetylation of all subtypes of histone H3 (total H3) exceeds the relative amount of this post-synthetic modification of the histone H4 complex (Table 1). Within histone H4 there was a continuous decrease of the relative quantitative amounts of [3 H]acetyl-CoA uptake from the monoacetylated subtype down to the tetra-acetylated form (H4.1 > H4.2 > H4.3 > H4.4). Little differences could be detected within all mafosfamide-treated livers, where the relative acetylation of H4.4 is higher than that of H4.3. Apart from this observation, decrease and increase in enzyme activities did not affect the acetylation patterns of histones H3 and H4 significantly (Table 1).

Influence of mafosfamide on the relative amounts of histone H1°

We observed the quantitative behaviour of the histone H1 complex, consisting of the subtypes H1A, H1B and H1°, in regenerating rat liver in the presence or absence of mafosfamide. Again we detected a significant transitory decrease of the relative amounts of histone H1° 16 h after partial hepatectomy in the absence of mafosfamide, which was part of a slow H1° decrease in regenerating rat liver starting with hepatectomy and lasting for about 60 h [29] (Fig. 4). By contrast, this transitory decrease in the relative amounts of H1° could not be observed in regenerating rat liver treated with mafosfamide. We also

estimated the slow and continuous decrease in amounts of H1^o under these conditions, but with temporary delay. At 23 h after partial hepatectomy, just before the onset of DNA synthesis in mafosfamide-treated livers, we detected a slight transitory reduction of H1^o amounts comparable with, but of less extent than, the decrease estimated in unaffected regenerating liver (Fig. 4).

DISCUSSION

The results presented here demonstrate that a maximum activity of histone acetyltransferase is also reflected by an elevated acetylation *in vivo* and that a consecutive transitory reduction of histone H1^o precedes the initiation of replication in regenerating rat liver in the presence or absence of mafosfamide. Application of mafosfamide at the start of liver regeneration causes a shift of the above-mentioned events for 7–8 h in the same sequence. At the time of maximum histone acetylation, 13 h after partial hepatectomy, we detected a transient reduction of enzyme activity in mafosfamide-treated livers in comparison with unaffected regenerating rat liver. This reduced enzyme activity in mafosfamide-treated liver was followed by a second and longer-lasting elevation of acetyltransferase activity 21 h after partial hepatectomy. Yet it is not clear whether the mafosfamide-induced reduction of enzyme activity is due to direct inhibition of acetyltransferase or to the suppression of certain mediators necessary for acetyltransferase activity. The phenomenon of the second maximum acetylation in mafosfamide-treated liver could be a regulation mechanism of the cell whereby sufficient acetylation could be achieved before the start of DNA synthesis. Another explanation could be a decreased concentration of mafosfamide due to metabolic decomposition and urinary excretion. In this second case the histone acetyltransferase and/or its mediators are no longer suppressed by this alkylating agent and therefore the activity of acetyltransferase increases with temporary delay.

Additionally, the longer-lasting total depression of replication in the presence of mafosfamide can not exclusively be due to cross-links, since it has been reported [19] that only very few DNA regions were affected by these mafosfamide-induced alterations of chromatin. This suggests that other events must be jointly responsible for this reduction of DNA synthesis to control levels.

The amount of histone H1^o is known to be altered during numerous cellular processes: it is increased in differentiating and non-dividing cells, and decreased in carcinoma cells and in several rapidly proliferating tissues [29–31]. Our observation of the transitory impairment of histone H1^o after maximum histone acetylation and the shift of both events in the presence of mafosfamide suggest that histone acetylation and histone H1^o reduction may be functionally related, since an acetylation-linked histone replacement has been reported on several occasions [32–34].

It was proposed [35,36] that acetylation of histones produces its effect preferentially by weakening the electrostatic histone-DNA interactions in the close proximity of the DNA linker region. It seems obvious that such modifications of solubility and stability of chromatin and its higher-order structures are of great importance in the initiation of specific cellular functions such as replication. This suggestion is in accordance with the findings of other authors proposing that the unwinding of active chromatin is necessary to enable enzymes to bind to DNA [37–39].

On the basis of our results and those in the above-mentioned papers [32–39] we suggest that a certain level of histone acetyltransferase activity resulting in enhanced acetylation *in vivo* may lead to a local destabilization of nucleosomal structure and consecutive replacement of histone H1^o. These events may

produce a local alteration of chromatin architecture sufficient to allow enzymes to bind to DNA and initiate replication.

One might argue that the maximum histone acetylation occurs as a result of a transcriptional process. On the contrary we have found that histone acetylation *in vivo* at the time of maximum RNA synthesis [40] is indeed increased compared with a sham-operated control, but it is significantly lower than acetate incorporation *in vivo* at the time points of maximum activity of histone acetyltransferase, occurring just before the onset of DNA synthesis (Fig. 3) [17].

However, a recently published paper suggesting that changes in histone acetylation might contribute to the control of histone H1 deposition, thereby regulating transitions in the folding of newly replicated chromatin [41], supports our speculation, proposing a possible role of histone acetylation and H1^o reduction for the initiation of DNA synthesis.

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