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Phosphorylation and chromatin mechanics: the central importance of substrate conformation in determining the patterns of HL-60 nuclear phosphorylation

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HL-60 nuclear autophosphorylation was studied *in vitro* under circumstances in which the conformation of chromatin was manipulated with both polyamines and DNAse-I. A general re-arrangement of the phosphorylation patterns occurred as polyamines were removed and nuclei were observed to expand. DNAse-I treatment reduced these phosphorylation patterns to a much simpler configuration indicating that the responding substrates were DNA-associated. It was concluded that substrate conformation was the main determining factor in the control of nuclear protein phosphorylation. These results suggest a method of general utility for the identification of truly nuclear proteins by the characteristics of their phosphate acceptor activity.

Protein phosphorylation/dephosphorylation is a major method of modulation of protein properties [1]. The balance between the phosphorylation and dephosphorylation of nuclear proteins has long been suspected to be one of the processes driving changes in nuclear conformation during the cell cycle [1-2]. This paper highlights the importance of the *converse*: the effect of chromatin conformation on protein phosphorylation *in vitro*. In these studies chromatin, kept in the well characterized conditions of "Buffer-A", in which DNA integrity and chromatin structure are mantained [3-9], was expanded and contracted by manipulating polyamine levels. While it is not entirely new to draw attention to the fact that the conformation of the substrate molecule may be extremely important [10] it was surprising to find that in isolated HL-60 nuclei, the conformation of the substrate molecules was the most important factor in the control of nuclear autophosphorylation.

MATERIALS AND METHODS

Cells: HL-60 cells were grown in RPMI 1640 media and harvested in late log phase in PBS (phosphate-buffered saline: 0.87%NaCl, 10mM Na2HPO4, pH 7.5).

Nuclear preparations: All buffers used in the preparation of nuclei are based on the basic salts/mercaptoethanol of buffer-A [3-4]. The base solution is 15 mM (0.1%) 2-

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mercaptoethanol, 15 mM NaOH, 60 mM KCl (pH is adjusted to 7.5 using HCl) and is referred to as "A". All washes were with 1 ml of solution for 5 min at 2°C. PBS-washed cells (19 mg wet weight of pellet) were washed once in A-HEPES-polyamines buffer (buffer-A made 15mM HEPES, 0.15 mM spermine and 0.5 mM spermidine) with 0.1 mM EDTA, 0.05 mM EGTA, 4 mM MgCl2, 0.1% bovine serum albumin and 40 µg/ml phenyl methyl sulphonyl fluoride (PMSF). The cells were then stripped of their membranes by washing them once with 0.05% Triton X-100 in the above solution except that (0.5%) bovine serum albumin and no PMSF were used. The samples were then washed in the appropriate ionic environment (see below).

Experimental conditions for structural comparisons: Three main ionic environments, generating three levels of nuclear condensation were used for incubations. These differed with respect to polyamine concentration and were H (high), M (medium) and L (low). Environments M and L had polyamines progressively removed by washing.

Environment H consisted of nuclei washed in A-HEPES-polyamines (buffer-A made 15mM HEPES, 0.15 mM spermine and 0.5 mM spermidine) containig 0.1% bovine serum albumin. For environment M nuclei were washed once in A-phosphate (buffer-A made 75 mM NaH_2PO_4, 0.1% bovine serum albumin) and once in A-HEPES (buffer-A made 15 mM HEPES, 0.1mM EDTA). Environment L was as for M conditions but washed one further time in A-HEPES to further extract polyamines. The condition L wash-extraction of the multivalent cations causes the nuclei to decondense so that they become intractable gels. For ionic environment H, the nuclei were finally suspended in A-HEPES-Polyamines, 0.1 mM EDTA, and for ionic environments M and L the final suspension solution was A-HEPES. HEPES was always used when buffers contained polyamines and phosphate was used as an aid in stripping polyamines as they are not readily removed [11]; DNAse-I (Boehringer Mannheim Gmbh) was used as a structural disturbing agent in some experiments. When DNAse-I was used, samples were made 5mM $MgCl_2$, 2µg of the nuclease were added and the tubes were incubated at 37°C for 30 min.

Autophosphorylation assays: An aliquot of nuclei or decondensed nuclear fraction (approximately 40 µg protein content as estimated by a modification of the method of Lowry [12]) was incubated in 60 µl of the buffer appropriate to its ionic environment, plus 5mM $MgCl_2$ and 40nM of γ-[^{32}P]-ATP (4000 Ci/mmol), 40ug/ml phosphatidylserine and 1.4mM free $CaCl_2$. Incubations were at 30°C for 3 min. Incubations were stopped by the addition of 1/3 volumes of 2% SDS, 10% glycerol, 62.5 mM Tris HCl pH 6.8, 5% 2-mercaptoethanol, 0.00125% (w/v) Bromophenol Blue, followed by incubation for 3 min in a boiling water bath. The lysate was incubated at room temperature for 30 min with 2 µg DNAase-I, 1 µg RNAase A. Despite the presence of SDS, the residual activity of the nucleases was enough to greatly reduce the viscosity of samples. Aliquots containing approximately 13 µg of protein were then analysed on polyacrylamide gels. Electrophoresis was performed following the method of Laemmli [13], using 12% SDS-acrylamide gels of 29/1 acrylamide/bisacrylamide.

RESULTS

Chromatin preparations, differing in their level of condensation, were phosphorylated *in vitro* by endogenous kinases utilizing γ-[^{32}P]-ATP as a source of phosphate.

The range of phosphorylation targets and the degree to which such targets were phosphorylated varied with the condensation state of the nuclei (Fig.1). This could be simply assessed from the changes in volume of the nuclear pellet centrifuged out under standard conditions. Stable, condensed nuclei (environment H) exhibited a relatively low

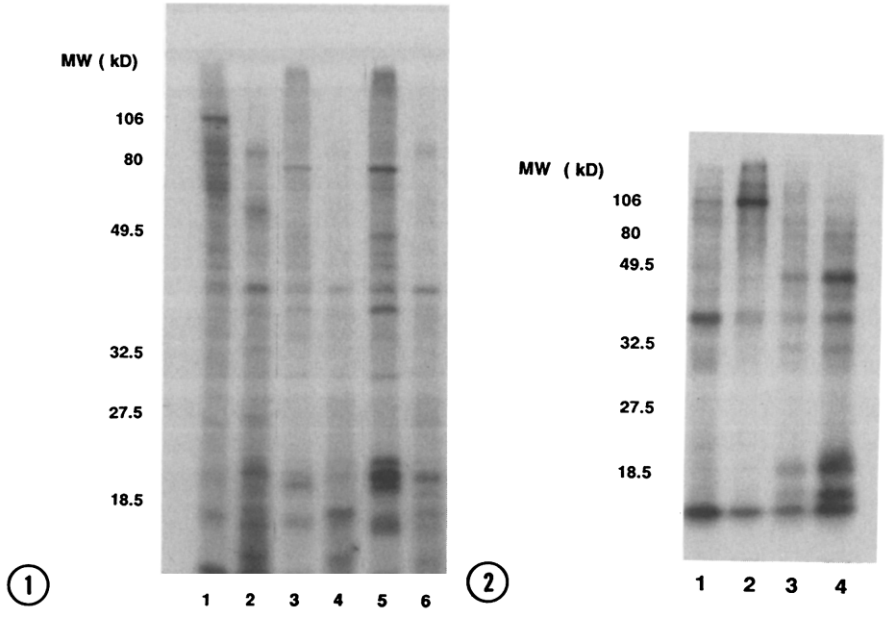

Fig.1. Effect of chromatin expansion and DNAse-I on the phosphorylation of nuclei by endogenous enzymes.
Nuclei were prepared in the three buffers described in the Methods section, corresponding to stable nuclei (H) and increasing levels of nuclear expansion (M and L). Lane 1: protein phosphorylation pattern obtained with nuclei prepared in buffer containing polyamines (H). Lane 2: the pattern obtained when nuclei prepared in buffer H are digested with 2µg DNAse I prior to phosphorylation. Lane 3: pattern obtained with nuclei prepared in environment M. Lane 4: the same nuclei digested with DNAse I before phosphorylation. Lane 5: phosphorylation pattern of nuclei prepared in environment L. Lane 6: the same nuclei subjected to DNAse I digestion prior to phosphorylation.

Fig.2. Reversibility of nuclear effects.
Stable nuclei were prepared in polyamines (environment H) or in a polyamine-free buffer (environments M and L) in order to cause their expansion. Nuclei were phosphorylated as described in the Methods. Lane 2 shows the pattern obtained by phosphorylating nuclei in an environment containing polyamines (stable, contracted nuclei). Lanes 3 and 4 show patterns obtained phosphorylating nuclei prepared in polyamine-free environments (M and L respectively). Lane 1 illustrates the pattern obtained when nuclei prepared in a polyamine-free buffer (L) are "recontracted" by readdition of polyamines and then phosphorylated. Note that removal of polyamines broadens the number of proteins phosphorylated by internal enzymes, and that the effect is reversible.

degree of phosphorylation except for one notable protein of molecular weight approximately 106 kDa (Fig.1). Forced expansion of the nuclei, caused by the removal of polyamine stabilisation and clearly apparent from the viscosity of the samples (particularly the nuclei exposed to environment L), had the effect of changing the pattern of phosphorylated protein targets, enhancing the degree of phosphorylation of some of the proteins. These proteins notably included species having molecular weights of about 78, 48, 46, 36, 21 and 16 kDa. However, the phosphorylation of other proteins, including the 106 kDa protein, was suppressed by nuclear expansion. The expansion of the nuclei could be reversed by adding polyamines back to the samples. Phosphorylation in "recontracted"

nuclei yielded a pattern similar to the one obtained when employing stable, contracted nuclei (Fig. 2). In particular the phosphorylation of the 106 kDa protein was recovered in "recontracted" nuclei confirming that its lack of phosphorylation in expanded nuclei was not due to protein loss. Coomassie-blue stained gels also confirmed that proteolysis and protein losses due to washing were not significant in these chromatin preparations (data not shown). The predominantly nuclear localisation of the proteins observed was deduced from the effects of DNase-I treatment. Under all conditions, DNase-I digestion of nuclei prior to their phosphorylation by the endogenous enzymes, produced completely different protein phosphorylation patterns (Fig. 1). These results indicate that the phosphorylations which were observed are linked to DNAse-sensitive structures.

DISCUSSION

Cytoplasmic contamination is always a problem with relatively "native" chromatin or nuclear preparations [14]. However, in the experiments presented in this paper, it is not necessary or appropriate to try to prove the "purity" of the preparations because the agents used are relatively nuclear specific in their effects. DNAse and low polyamine levels are expected to have a variety of specific side-effects in non-nuclear systems but, collectively, they would be expected to have their major effects in a DNA-containing system. Polyamines are well known to affect protein phosphorylation in cellular systems [15]. Their effect seems to be achieved through conformational changes of the substrates rather than by means of direct action on the kinases [10; 15-17] and it has previously been suggested that these compounds might influence the phosphorylation of chromatin-associated proteins by altering chromatin structure [18]. In these previous studies, however, the polyamine effects have been largely confined to the observation of gross, overall stimulation of phosphorylation in crude nuclear preparations [18-19]. In the HL-60/buffer-A system the polyamine-mediated conformational changes are also best interpreted in terms of different levels of condensation of the chromatin as the greatest changes in autophosphorylation occurred when expansion of the nuclei was obvious. DNAase-I further modulated the autophosphorylation patterns under all conditions and this indicates that almost all of the phosphorylation targets were truly DNA-associated at least at the time of phosphorylation (DNAse-I effects through actin-binding [20-21] would only be expected at stoichiometric, ie high, levels of DNAse-I).

Noteworthy in these comparisons between condensational states of chromatin were not only the many bands that increased in phosphorylation with chromatin expansion but also the proteins whose level of phosphorylation decreased. The latter might possibly be proteins that naturally drive decondensation *in vivo*. This is because they might be expected to be in their most susceptible configuration in tightly condensed chromatin and

those proteins that naturally drive condensation might be expected to be most susceptible in the decondensed state. These facts suggest the operation of the usual principles of negative feedbacks found in most control systems.

At a practical level, this study also suggests an approach to a common problem of classification of proteins in the analysis of chromatin. The problem is posed by the large variety of potentially "non-histone" proteins commonly found in nuclear preparations that may be weakly nuclear-bound or of doubtful nuclear origin. In many cases it is difficult to be sure that these proteins are nuclear in their usual localisation and function [14]. However, if these proteins can be phosphorylated, acetylated or methylated in nuclear preparations and agents such as DNAse-I and other nucleases strongly modulate their modification then this is at least *prima facie* evidence that the protein is not merely an adventitious cytoplasmic contaminant.

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